Unraveling the mechanistic effects of Pulsed Electric Magnetic Field (PEMF) stimulation towards directing stem cell fate and function: A tissue engineering perspective

Author links open overlay pane lGreeshma Thrivikraman[abd]Sunil KumarBoda[ae]BikramjitBasu[ac]

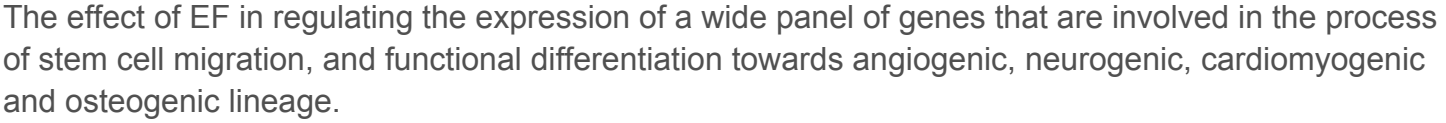
Abstract

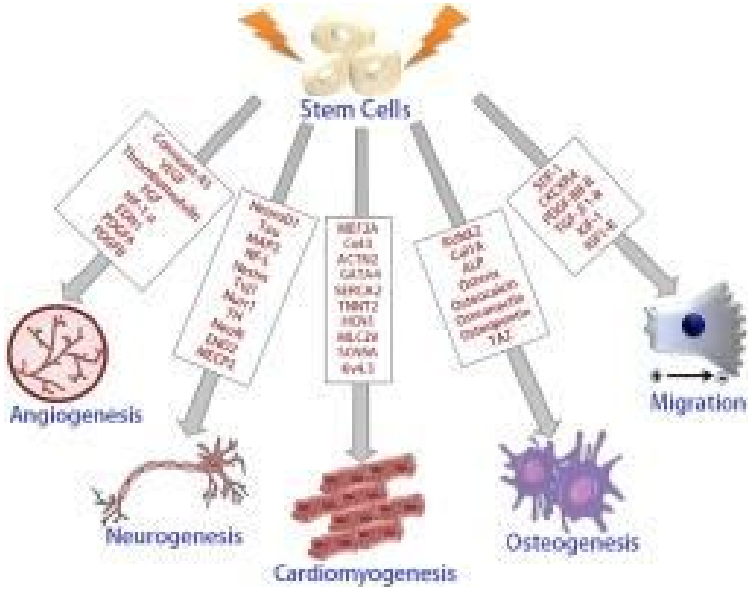
Electric field (EF) stimulation can play a vital role in eliciting appropriate stem cell response. Such an approach is recently being established to guide stem cell differentiation through osteogenesis/neurogenesis/cardio myogenesis.

Despite significant recent efforts, the biophysical mechanisms by which stem cells sense, interpret and transform electrical cues into biochemical and biological signals still remain unclear. The present review critically analyses the variety of EF stimulation approaches that can be employed to evoke appropriate stem cell response and also makes an attempt to summarize the underlying concepts of this notion, placing special emphasis on stem cell-based tissue engineering and regenerative medicine.

This review also discusses the major signaling pathways and cellular responses that are elicited by electric stimulation, including the participation of reactive oxygen species and heat shock proteins, modulation of intracellular calcium ion concentration, ATP production and numerous other events involving the clustering or reassembling of cell surface receptors, cytoskeletal remodeling and so on. The specific advantages of using external electric stimulation in different modalities to regulate stem cell fate processes are highlighted with explicit examples, *in vitro* and *in vivo*.

Graphical abstract





Keywords

Exogenous and endogenous electric field (EF)
Stem cell response
Cell surface receptors (CSR)
Reactive oxygen species (ROS)
Heat shock proteins (HSP)
Intracellular calcium $[Ca^{2+}]_i$

1. Introduction

It is well known that nerve, muscle and glandular tissues make use of endogenous electric fields (EF) to transmit electric signals/impulses [1], [2]. It is also well established that endogenously generated bioelectric currents play a critical role in important biological processes including embryogenesis, wound healing, tissue repair and remodeling as well as normal growth of organisms [3]. Endogenous EF exists in both the cytoplasm and extracellular space. Such EF can vary in strength from as small as a few mV/mm to hundreds of mV/mm [4]. It may be noted that electric stimulation of cells has been in practice for quite some time now. In clinical settings too, EF treatment is being extensively used, especially to revive the damaged or disabled tissues in the neuromuscular system (CNS – brain and spinal cord; PNS – sensory and motor neurons) as well as to accelerate healing of injured musculoskeletal tissues such as bone, ligament and articular cartilage. Taken together, such biophysical mechanisms suppress the progression of bone diseases like osteoarthritis and osteonecrosis [5], [6], [7]. Furthermore, EF is being proposed as a viable therapeutic option to minimize pain, to overcome tissue malfunction/impairment, to reduce muscle spasm, and to promote overall tissue/organ function [8]. Similarly, direct deep brain stimulation is reported to be beneficial in treating Parkinson's disease, by ameliorating symptoms through stimulation of basal ganglia [9].
The treatment of biological systems/cells with EF can evoke favorable biochemical and physiological responses, provided that the exposure duration and EF strength are within tolerance limits [10]. However, the predominant mechanism of EF interaction with biological systems still remains a mystery. Nevertheless, the biophysical changes upon EF exposure can be triggered at the cell surface, affecting membrane protein functions like enzyme activity (Na^+/K^+ATPase and

Ca^{2+} ATPases), membrane-receptor complexes and ion-transporting channels by altering the charge distribution (i.e. the conformation) on these biomolecules (Fig. 1)[11], [12]. Often, it is believed that a similarity exists in the signaling pathways triggered by mechanical stress and electric field [13].

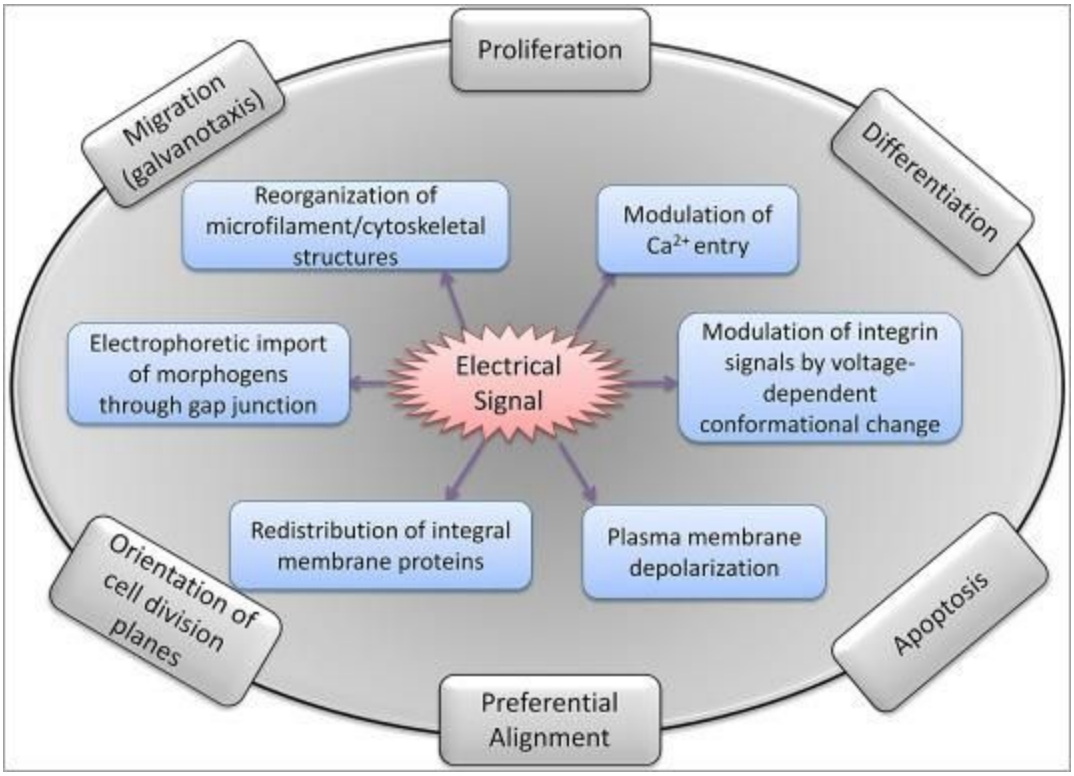


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Fig. 1. Bioelectrical control mechanisms at cellular level (clock-wise): Electric field (EF)modulates levels of intracellular calcium, a secondary messenger that drives numerous cellular processes and signaling cascades that govern proliferation and differentiation of stem cells. Endogenous EF guided embryogenesis implicates the key role of integrin receptors, a group of transmembrane linkers of the cell membrane to the extracellular matrix (ECM). The integrin receptor conformation is sensitive to alterations in membrane potential, thus affecting differentiation. Tumor treating oscillating EFs induce cell apoptosisvia plasma membrane depolarization. Numerous cell surface receptors (CSRs) such as epidermal growthfactor-like receptor (EGFR) undergo redistribution on the cell surface under the influence of an applied EF. This leads to a preferential alignment of the cell axis and orientation of cell division planes during mitosis. The import of morphogens and neurotransmitters through longitudinal gap junctions driven by electrophoretic mechanisms as in embryonic morphogenesis to the three germ layers is central to the migration of stem cells. Electrotaxis/galvanotaxis of cells occurs by reorganization of the cytoskeletalstructures such as tubulin and actin microfilaments, wherein actin nucleation and polymerization is induced in the direction of the applied EF by actin-related protein (ARP2/3) complex. Adapted from Ref. [18] Copyright ◦ 1999, FASEB Journal.

In order to realize the underlying phenomenon at the cellular level, one needs to determine first, whether the electric field exerts its effect directly on the cell or indirectly through alterations of physical or chemical factors in the extracellular environment. There are three probable lines of action by which external EF can exert its effect. a) The EF may act intracellularly by influencing the

movement and concentration profiles of charged cytoplasmic molecules [14]. b) It may perturb the transmembrane potential (TMP) that can alter the membrane responses and can activate growth-regulating ion transport across the plasma membrane [15]. c) It may also act along the plasma membrane, causing an electrophoretic accumulation of surface molecules or by modulating the conformational states of membrane proteins [16]. Such conformational changes are mainly induced by the interactions of protein dipole moments with electrically modulated membrane potentials [17]. The motivation for this review is to inspect various established approaches for the electro-manipulation of stem cells, in particular reference to the predominant mechanisms guiding stem cell response. Though such molecular mechanisms of EF interaction with stem cells are not explicitly understood, some compelling arguments are presented in the subsequent sections to explain the effects observed *in vitro.* The major objective of this review is to bring forth to the tissue engineering community, the need for a detailed investigation of the molecular mechanisms of EF stimulation of stem cells, which would eventually provide a rational starting point for future pre-clinical and clinical studies.

In this review, the different facets of electrical stimulation in the context of guiding stem cell fate and function are discussed. This review encompasses the physiological origin of endogenous bioelectric fields and the experimental approaches to simulate endogenous electrical signals by exogenous EF stimulation. Furthermore, the modulation of stem cell proliferation, migration and differentiation to multiple lineages (osteogenic, neurogenic, cardiomyogenic and angiogenic) by manipulating EF stimulation parameters are exemplified. Also, a critical analysis of the possible mechanisms of EF dictated stem cell response such as biochemical signaling pathways, calcium transients, cytoskeletal reorganization, ATP synthesis, reactive oxygen species and heat shock proteins is provided. Finally, the utility of exogenous EF for deep brain stimulation, cardiac pacing and defibrillation, *in vivo* is illustrated.

2. Endogenous vs exogenous EF

Endogenous EFs are considered to be essential for maintaining cellular homeostasis and are invoked in many biological events, from embryonic development to healing of the wounded tissues. EFs of detectable magnitude have been reported to occur in tissues and embryos of different origin, such as in Xenopus, chicken, and mouse [19]. Endogenous EF of around 20 mV/mm were measured in a 2–4 days old chick embryos and disruption of such field affected tail development structures. A similar EF was recorded in axolotl embryo, interference of which caused developmental abnormalities during neurulation, but not gastrulation [20]. Such examples highlight the importance of endogenous EF during embryo development. From the regeneration point of view, studies in cultured *Xenopus* embryonic neurons indicated striking orientational effects in such a way that, applied EFs induced neurite sprouting and promoted the turning of the growth cones of extending neurons [21]. Hinkle et al. demonstrated that a low strength EF can induce amphibian neurite growth towards the negative pole, *in vitro* as well as control the bipolar orientation axis of developing muscle cells from spherical myoblasts [22]. Further, in the context of endogenous EF for wound healing and tissue regeneration, pharmacologically manipulated endogenous EF generated from rat corneal wounds regulated the orientation and frequency of cell division testifying the role of wound induced EF in tissue defect healing [23]. All these published reports testify the role of endogenous EF on healing of tissue defects. Based on these groundbreaking observations, the devices generating pulsed electromagnetic fields have been approved by the FDA [24]. In a landmark study, Borgens et al. detected changes in current pattern and density at the dissected bone site, while steady ionic

(electric) current was recorded away from bone defects created in the metatarsals of weanling mice. The observed “fracture currents” decayed depending on the extent of bone injury and they were distinct from the stable persistent current in the healthy bone, which were driven majorly by chloride ions and to a minor extent by sodium, magnesium, and calcium ions [25]. The biological role of endogenous EF has also been implicated in long-range protein interactions, in electron transfers of chemical reactions and consequently influencing chemical reaction kinetics in diseased cell state such as cancer and in intercellular interactions [26].

Exogenous EFs are non-endogenous bioelectric fields, generated from external power sources and typically applied to biological cells/tissues via electrodes. Currently, different kinds of electrical stimulation modalities are being applied in several experiments, *in vitro*. These include direct current (DCEF), pulsed (PEF), alternating current electric field (AC EF), oscillating magnetic flux induced electrical stimulation and so on. Of them, DCEFs have gained significant attention among biomedical scientists and engineers, mainly for *in vitro*stimulation studies, as it can alter the migration and cell shape, apart from influencing the viability and proliferation of numerous cell types [27]. Also, there are other ways of treating cells with EF such as capacitive coupling (CC) and inductive coupling (IC). Each of them will be briefly described in this section along with some of the commercially available electrical stimulators. Usually, *in vitro* DC EF setups are constructed from petri dishes or cell chambers with conductive electrodes (e.g. graphite, platinum wires or stainless steel) being placed directly in culture medium (Fig. 2) [28]. Moreover, the electrodes can locally deliver the applied current between the anode and cathode accurately along a specified direction [29]. Another experimental set up to apply EF to the cells or tissues is to custom-design an isolated chamber connected via agar salt bridges to external Ag/AgCl electrodes immersed in Steinberg’s solution (Fig. 2). Such a configuration can isolate the EF exposed cells from reactive faradaic products of electrolysis (hydrogen peroxide, hydroxyl and superoxide ions, or other free radical intermediates) generated due to the redox reactions occurring at the electrode-electrolyte interface [30]. In addition, the adsorption of proteins on the electrodes leads to a reduction in the magnitude of the electrical stimulus. This occurs because the flow of electrons or current is impeded by the adsorbed proteins and this eventually limits the effectiveness of DCEF [31]. On the other hand, capacitive coupling (CC) and inductive coupling (IC) (Fig. 2) are non-invasive ways of exposing cells to EF. CC devices consist of two parallel disc-electrodes coupled to the culture, with the lower electrode placed at the bottom of the culture dish and the upper electrode placed above the medium, leaving an intermediate air gap [32]. The electrical stimulation occurs through the induced EF, mediated by the transfer of electrical energy from the capacitor plate to the targeted cell monolayer [33]. In IC technique, an EF is induced perpendicular to the axis of a time-varying magnetic field caused by a change in current flow through a coil of wire or solenoid [34]. The induced voltage/electric field parameters are governed by Faraday’s induction law and depend on the frequency characteristics of the applied magnetic field and the impedance characteristics of a cell or a biological system [35]. However, the observed cellular effects in such cases are a superposition of both electric and magnetic fields. To address this concern, Hess et al. developed a culture platform based on transformer-like coupling principle, to apply EF without any interference from magnetic field to direct osteogenic differentiation in human mesenchymal stem cells (hMSCs) [36].

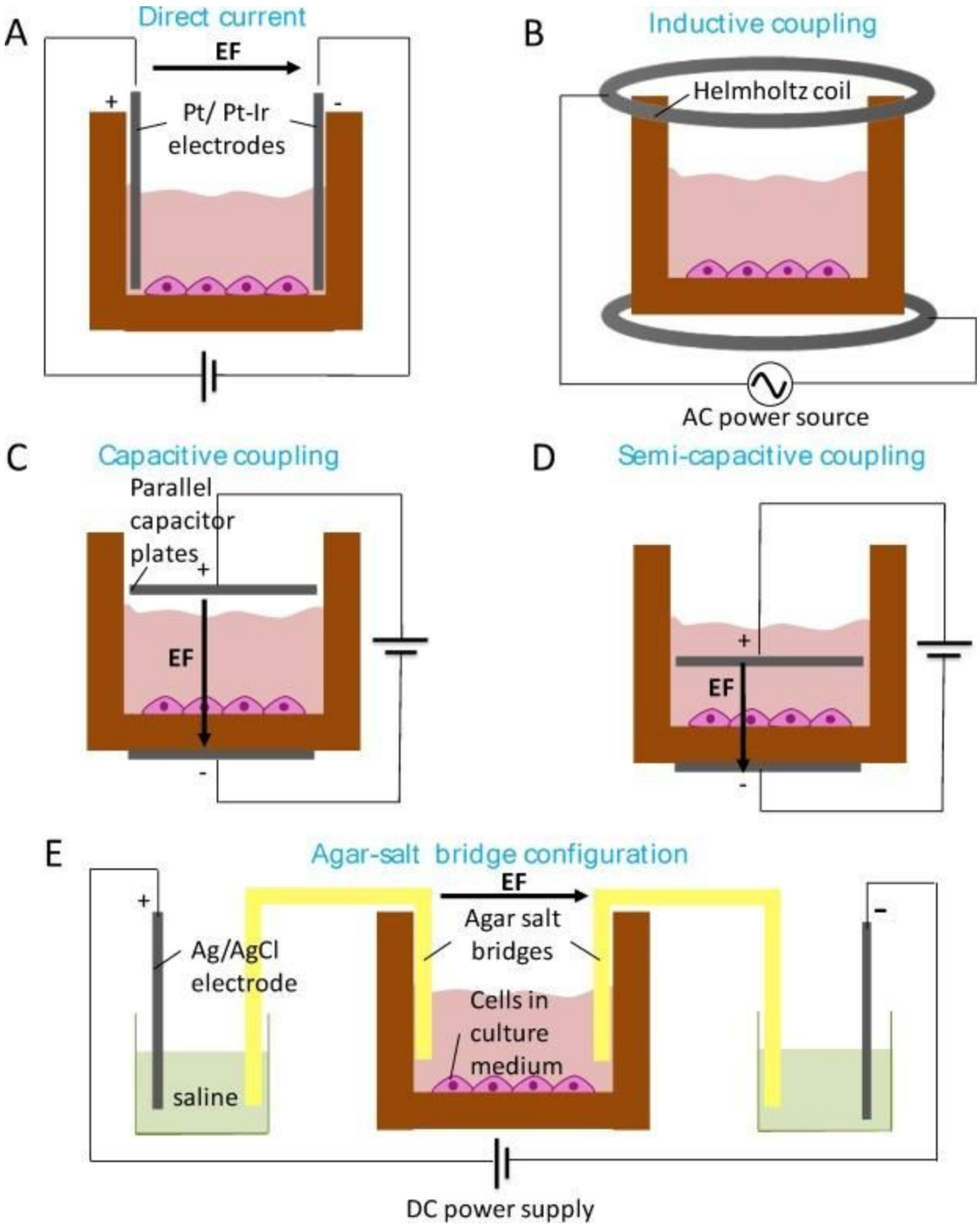

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Fig. 2. Schematic representation showing various approaches for EF application in *in vitro* cultures – (A) Direct current (DC) stimulation utilizes two parallel electrodes coupled directly by immersion into the culture medium and connected to a power source; (B) Inductive coupling (IC) creates EF from oscillating electromagnetic fields generated by passing alternating currents through a pair of Helmholtz coils; (C) Capacitive coupling (CC) produces EF by using two metallic/conducting plates placed above and below the cell culture dishes without contact with the culture medium, while (D) one

of the plates (usually the top plate) is immersed into the culture medium in the case of semi-capacitive coupling; (E) In the agar salt bridge configuration, the electrodes are immersed in electrolytes in separate chambers and connected to the cell culture dish through an agar salt bridge for maintaining EF.

It is often challenging to apply EFs to monolayer cultures, 3D cultures, tissue engineered constructs or embryos, especially during the long term culture experiments. In some cases, such long duration high throughput experiments demand bulky circuits connected with multiple wires running into and out of the incubators. In order to address these challenges, miniaturized stimulation device consisting of circuit layouts on minute electrical boards and 3D-printed cell culture chambers with on-board integrated circuits (ICs) and biocompatible cell arrays were proposed, so as to examine the effects of different stimulation parameters on cell behavior [28].

It is also worthwhile to emphasize the disadvantages of using electronic circuits with fixed resistances/impedances, as the intrinsic impedance of cells and the cell medium can change as a result of EF induced cellular responses. Rather, such studies require variable voltage or current outputs that can compensate for the fluctuations in biological responses. Often, arbitrary choice of EF parameters leads to incomplete knowledge of the fundamental pathways that mediate EF interactions with cells. Thus, for the correct interpretation of the studies, a thorough optimization of electrical stimulation parameters, such as field strength, time duration and frequency, would be required so that, the cell can bypass through its initial, passive state to reach the threshold level for active response [37], [38]. Notably, since the cells are electrically shielded by the cell membrane, the extent of shielding is frequency dependent in an alternating field and depends on the rise time and pulse duration [10].

A number of electrical stimulation set ups and devices are commercially available for *in vitro*and *in vivo* studies, respectively [39]. Mobini et al. used DC electrical stimulation chambers with L-shaped Platinum (Pt) electrodes fixed onto the lid of standard 6-well plates dipping into the culture wells [40]. Using this set up, DCEF of 100 mV/mm was applied to rat adipose-derived mesenchymal stem cell cultures to elicit osteogenic differentiation over 1 week in culture. In another study, a 24-well plate flanked by capacitor circuitry on electrical boards was used to elicit the synchronous contraction of C2C12 skeletal muscle cells [41]. Such a setup also contains semi-circular platinum electrodes, immersed into the culture medium with end electrodes connected to the electrical boards. Another commercially available electrical stimulation device for chronic EF stimulation is the C-Pace EP Culture Pacer by IonOptix. It is a multi-channel biphasic EF stimulator with output voltages of 0–40 V, 0.01–100 Hz and pulse duration of 0.4–24 ms. This set up has been applied predominantly for promoting contractile activity and external pacing of cardiomyocytes in 2D as well as 3D cultures [42]. In parallel to the C-Pace EP stimulator, Xiong et al. custom designed a miniaturized single channel electrical stimulation set up, which can accommodate multi-well plates within the stimulation board and provides a greater variation in the frequency and pulse wave forms [28]. For pre-clinical studies, the implantable EF stimulators are enclosed in a hermetic seal or epoxy resin to avoid electrochemical reactionswith the body fluids. These devices/modalities are discussed in a later section on *in vivo*effects of electrical stimulation, including deep brain stimulation and cardiac pacing.
The choice of electrode material is a crucial parameter that can influence the therapeutic efficacy of electrical stimulation in biological tissues. By using electrodes for ES, one can have a local control on

the magnitude of the electrical field. Most of the electrodes create and inject/transfer charge through the production of electrons by oxidation at the anode. However, in physiological systems electrical charges are transported via ions present in the electrolyte. In an electrochemical cell, charge transfer from electrons to ions occurs at the electrode–electrolyte interface via Faradic or Non-faradic electrochemical reactions and the formation of capacitive electrical double-layers. The charge-injection mechanism is dependent on the nature of electrode material, electrolyte and electric potentials at the electrodes. For both *in vitro* and *in vivo* stimulation experiments, the electrode material has to be chosen in such a way that it has acceptable biocompatibility without inducing the irreversible Faradaic corrosion reactions in the culture medium during ES [43]. Based on the chemical nature of the electrode, there exists an intrinsic charge injection limit, which determines the voltage output at the electrode surface. Beyond this threshold voltage, irreversible electrolysis of water occurs, which can lead to cellular damage by the dissolution of electrode material, pH variation and generation of undesirable reactive chemical species as well as alteration of cellular metabolic rates caused by Joule's heating [44]. The choice of a potential electrode material varies, depending on its use. Notwithstanding, electrode materials with low impedance, high charge injection capacity, large reversible charge transfer and long term stability are highly desired to achieve effective and safe electrical stimulation [45]. To this point in literature, different strategies are applied to limit the corrosion rate, such as cathode and anode protection by the formation of passivation layers and barrier coatings over the electrode surfaces. Conducting polymers, such as polypyrrole(PPy), polyaniline (PANI) and polythiopene (PT) are being used in recent years as corrosion protective coatings for iron/steel [46]. Another issue is that charge injection is directly proportional to the electrode area. Therefore, lower the electrode area, lesser is the charge injection leading to undesirable greater electrode impedance. This can be overcome by roughening the electrode material, which increases the real surface area, without modifying the geometrical surface area of the electrode [47].

Currently, a number of materials are being used for fabricating biomedical electrodes for *in vitro*, pre-clinical and clinical experiments, namely Carbon (Graphite rods), Platinum (Pt), Iridium (Ir), Gold (Au), Titanium (Ti), Tungsten (W), platinum-iridium alloys, and titanium nitride, Stainless steel (SS), Indium Tin Oxide (ITO) etc [48]. Among them, Pt is the preferred electrode material for *in vitro* cell stimulation, neuroprostheses, cochlear implants, deep brain stimulation and retinal implants. This is due to its electrochemical stability, inertness and corrosion resistance, leading to its limited reactivity in biological environments. However, for *in vivo* applications, Pt is alloyed with Iridium to make the electrodes mechanically strong and implantable deep into the tissue for EF stimulation. Likewise, 316 LVM stainless steel is also commonly used as it has less likelihood of mechanical failure [49]. Even though Au and Pt are more resistant to corrosion than stainless steel, they are more prone to mechanical failure. However, during ES, the chromium oxide barrier layer in stainless steel can form cracks, allowing the oxidation of the iron underneath, resulting in electrode decomposition. Thus, for very long duration or high intensity stimulation protocols, the use of platinum or platinum-iridium electrodes would be preferred [50]. Despite having a lower polarization resistance (R_p, the electrode resistance to chemical reaction) than titanium and titanium electrodes, only SS can maintain constant current over 90 s of ES, while the polarization of the culture medium and the shielding of charges occurs on Ti and TiN electrode (highly resistant to Faradaic reactions) [4]. It was observed that highly capacitative and corrosion resistive carbon electrodes had the best charge injection and transfer features for use in electric stimulation of cardiac and neuronal cells [51], [52]. However, the graphitic carbon rods suffer from poor mechanical strength and protein adsorption, that

disrupt the uniformity of EF intensity over time in culture [51]. This necessitates the cleaning of the electrodes between successive stimulation protocols. Lately, standard corrosive electrode materials are being replaced by non-metallic, biocompatible and electroconductive materials [53]. With organic conducting polymers, the charge transfer capacity along with their polymeric nature enables a greater affinity and connection with biological systems [54]. Recently, carbon nanotube (CNT) surfaces have been widely investigated as effective electrodes for neural interfaces, owing to their phenomenal properties such as large surface area, high electrical conductivity and mechanical toughness as well as excellent charge injection ability and decreased interfacial electrode impedance, along with their capability to support neuronal adhesion and growth [55].

Altogether, scientific investigations over the years have explored several promising materials to benefit the challenging requirement of ES both *in vitro* and *in vivo*. Although apparent, the electrode materials must possess better mechanical properties and higher charge injection capacity for EF stimulation *in vivo*. In comparison to EF stimulation of cell monolayers, electrodes are prone to mechanical failure or bending during insertion into tissues as well as the larger reduction in charge injection capacity *in vivo* compared to *in vitro* EF stimulation [56]. The advent of micro and nanofabrication technology has opened up countless opportunities to produce novel electrode systems ranging from ultra flexible transparent electrodes, micro/Nano needle electrodes, microelectrode arrays, metal nanomesh electrodes, nanowires, patterned metal grids and so on [57], [58], [59]. Table 1 presents a list of electrode materials that are currently in use for *in vitro* and *in vivo* stimulation of cells and tissues, respectively. A brief survey of the electrode materials revealed that most of the pristine metal or single element electrodes suffer from mechanical failure limiting their application for chronic and deep tissue stimulation, *in vivo*. Also, the corrosion resistance of alloys and metallic electrodes results from the formation of a surface passivation protective layer.

Table 1. Electrodes for electrical stimulation of cells and tissues.

Electrode material	Cells/Tissue stimulated	Advantages	Disadvantages	Reference
Carbon rods	2D and 3D cultures of cardiac cells	High current injection efficiency; Can be applied for 2D and 3D culture stimulation in bioreactors	Poor mechanical strength; Protein adsorption on carbon electrodes can affect the uniformity of EF	Tandon et al., 2006 [51] Tandon et al., 2009 [60]
Carbon film	2D cultures of neuroblastoma and Schwann Cells	Porous carbon film can be simultaneously used as substrate for cell culture as well as electrode for vertical EF stimulation	Fragile in nature	Jain et al., 2013 [52]

Platinum (Pt)	2D and 3D cultures of C2C12 muscle cells	Highly inert, preferred for chronic stimulation; Can generate reproducible and uniform EF	High cost and poor mechanical stability	Ahadian et al., 2012 [61]
Platinum-Iridium (Pt-Ir) alloys	Deep brain stimulation and extracochlear implants	Alloying Pt with Ir improves charge carrying capacity and mechanical robustness for deep tissue stimulation	Fabrication by electrodeposition is costly and time-consuming; Non-uniform coatings	Ison et al., 1987 [62] Petrossians et al., 2016 [63]
Gold	3D cultures of Cardiomyocytes	Array of gold electrodes deposited on epoxy resin for cardiac stimulation; Non-toxic and non-reactive at low voltages	More corrosive than Pt; Mechanically weak necessitating alloying with other metals and cost ineffective	Radisic M. 2016 [64] Blume et al., 2013 [65]
Titanium; titanium nitride	Cardiomyocytes in Matrigel scaffolds	Unreactive for moderate voltages, mechanically strong and biocompatible	Low charge injection efficiency; Can corrode under prolonged usage	Tandon et al., 2011 [66]
Tungsten	Brain stimulation	Preferred for deep tissue stimulation; Non-reactive during DC stimulation due to surface passivation oxide layer	Undergoes dissolution during pulsed stimulation; Not suitable for chronic stimulation	Tehovnik EJ. 1996 [67] Stevenson et al., 2010 [50]
Tantalum	Pacemaker electrodes	Ta is corrosion resistant due to Ta_2O_5 passivation layer formed on Ta surface; Can be safely used even as anode	Rarely used as alloying agent as it imparts brittleness to other metals except steel	Levine et al., 2006 [68] Johnson et al., 1977 [69]
Nichrome	Peripheral nerve stimulation and recording	Nichrome wire braids have good mechanical strength and biocompatibiity	Chronic stimulation can pose toxicity from the leaching of Ni and Cr	Jellema et al., 1995 [70] Kim et al., 2013 [71]
Nitinol	Endovascular neural interfaces	Being a shape memory alloy, it can be used to penetrate the blood brain barrier and record neural signals	Dissolution of Ni from nitinol can cause potential toxicity to tissues	Wong et al., 2016 [72]
Stainless steel (316L)	Endocrine glands in rats and mouse embryonic stem cells; 2D culture of hMSCs	Mechanically strong and electrode surface is protected by a passive iron oxide layer	Dissolution of iron at high positive voltages can lead to ROS generation and toxicity; This limits the usage of stainless steel for neural stimulation and prostheses	

3. Effects of EF on stem cell niches

Stem cells are the most promising candidates in the field of tissue engineering and regenerative medicine due to their ability to regenerate and repair damaged tissues at the sites of injury [83]. Stem cells exhibit characteristic features such as high proliferative capacity (in an undifferentiated state) and the potential to differentiate along one or more lineages under appropriate culture conditions. They are found in a complex and dynamic microenvironment called niche, which comprises of supportive cells as well as components of the extracellular matrix arranged in a three-dimensional topography [84]. Stem cell niche dictates the fate of stem cells through unique signaling environment

imposed by modulating the matrix stiffness, by presenting immobilized signaling molecules in a defined manner, by establishing cell-cell contact, by the creation of cytokine gradients and also through multitude of other physical factors, such as fluid shear and mechanical stress, partial pressure of oxygen and temperature during culture [85], [86]. When a stem cell undergoes division in a complex physiochemical environment, each new daughter cell can choose to either remain a stem cell or become a functionally specialized cell such as a muscle cell, a bone cell, or a brain cell [87]. As stem cell activation, homing and differentiation are central to tissue development, homeostasis and repair, the niche preserves the cells in a quiescent and metabolically less active state to replenish them and balance their availability and migration towards sites of injury [88]. A series of different factors play important roles in stem cell fate decisions. Understanding the harmony in the spatial and temporal interaction of signals from the matrix microenvironment is an essential prerequisite for the application of stem cells in regenerative medicine to create desired complex, functional tissues. By precisely tuning some of the physical factors, such as ECM geometry, topography, elasticity, electrical or mechanical and biochemical signals, localized stem cell differentiation can be achieved, without the use of potentially toxic biochemical reagents or soluble factors.

As it is well known that endogenous EFs can stimulate tissue regeneration and that electric fields induce stem cell recruitment to the wound site, evidences suggest that local electric fields greatly influence stem cell fate [89]. Thus, regardless of the various strategies that are at work to activate stem cell function for tissue formation/remodeling, EF has tremendous significance in stem cell therapeutics to maintain, regenerate, or heal tissues [90], [91]. It is well known that stem cell behavior is regulated by the anatomical, physiological and functional microenvironment known as niche that they reside in. Such cellular environment constitutes the niche around stem cells. Similarly, stem cell recruitment to the wound site is regulated by local endogenous electric fields. This is hypothesized to constitute the electrical niche that enables stem cell dictated wound healing [92]. The electrical component of stem cell niche can affect membrane depolarization leading to plasticity of stem cells by alteration of the membrane voltage. Bioelectric signaling prevailed over biochemical signaling as illustrated by the transdifferentiation or increase in the stemness of hMSC derived adipocytes and osteoblasts upon membrane depolarization by Na^+/K^+ ATPase inhibitor [93]. Thus, the electrical niche around stem cells can significantly enhance their plasticity and guide differentiation to various lineages. Based on the electrophysiology of the different body tissues, it is also possible to manipulate stem cell differentiation towards specific lineage by mimicking the electrical conductivity and electrical activity of the tissue in question. Also, the exogenous stimulation protocols must be designed to have a close resemblance to endogenous electrical currents and fields in the native tissue. For instance, pulsatile electrical stimulation of biowires embedded in 3D hydrogel matrix was shown to induce maturation of stem cell derived cardiomyocytes in a frequency dependent manner, as evident from the phenotypic expression of contractile proteins, electrophysiology and calcium oscillations [94]. The following sections are intended to further exemplify the distinct effects of EF exposure on stem cells, based on their self-renewal (i.e., sustained replicating capacity), motility (i.e., migration), and multipotency (i.e., capacity to differentiate into multiple cell types). A detailed account of the EF stimulation effects on stem cell proliferation, migration and differentiation to multiple lineages along with the mechanisms of stem cell activation by exogenous EF are presented in the following sections.

3.1. Stem cell proliferation

The ES effects on cell proliferation are widely reported and it includes both inhibitory and stimulating effects, depending on the cell type and exposure conditions [95]. In several instances *in vivo*, cell divisions occur in the presence of DC physiological endogenous EF, such as, during morphogenesis of mammalian embryo, wound healing, or tumor formation. Similarly, a dose dependent increase in proliferation was observed in *in vitro* cultures, for various cell types (HL-60 leukemia cells, Rat-1 fibroblasts and WI-38 diploid fibroblasts) exposed to low frequency EF [96]. Under identical exposure conditions, unexpected DNAstrand breaks in later hours were noticed suggesting that the EF exposure caused a temporary mitogenic effect, followed by a loss of DNA integrity. Hence, this study is an apt example validating from a pathogenic view point, that short-term exposures (<12 h) induce growth stimulation whereas prolonged exposures (>24 h) impede the advancement of the cell cycle by causing DNA damage [96]. Thus, exposing cells to EF for shorter duration is a viable option to harness its utility to enhance proliferation and differentiation.

In contradiction to the study by Wolf et al. [97], another group came up with the inhibitory effects of intermediate frequency AC fields, termed as tumor treating fields (TT Fields) in preventing cancerous cell growth, *in vitro* and *in vivo*[98]. In a study aimed at promoting proliferation of ASCs, a 448 kHz EF stimulus, which is currently used in electrothermal capacitive-resistive electric transfer therapies, could activate proliferation without compromising the multipotentiality of ASCs to subsequently differentiate towards adipogenic, chondrogenic or osteogenic lineage [99]. In the same way, numerous reports in literature validate the effect of EF in prompting higher proliferation rates in adult stem/progenitor cells, though the exposure parameters and treatment protocols (intensity, type of EF, pulse duration, exposure time) were different in these experiments [82], [100], [101], [102], [103], [104], [105]. In fact, to illustrate the varying effects of different forms of ES on proliferation, Griffin et al. exposed hMSCs to DC, capacitive coupling (CC), pulsed electromagnetic field (PEMF) and degenerate wave (DW) EF [106]. It was observed that DW led to maximum cell proliferation and minimal apoptotic cell death and other cytotoxic effects relative to other waveforms [105]. The functional electrical stimulation treatment of neural stem cells (NSCs) enhanced the production of EGF/bFGF and these growth factors subsequently exerted their effects on NSC proliferation [107].

Although, the conventional biochemical factor based strategies certainly predominate in effectively controlling cell growth, these soluble factors supplemented in the culture medium have shortcomings in terms of the quality, source, concentration of the utilized factors, localized delivery and so on [108]. As a result, recent studies advocate the combinatorial strategy of coordinating well-defined interactions between physical and biochemical cues to influence the cell fate determination [85]. One possible explanation for the enhanced proliferation is the augmented convection of nutrients and soluble factors by electrokinetically driven flow [109]. The applied EF can enhance the effective mass transport by either electrophoresis or electroosmosis. Under an applied EF, both these phenomena can aid in convection based transport of both charged and uncharged molecules. Moreover, EF causes a variety of proteins to re-distribute asymmetrically, which in turn plays a critical role in determining the level of symmetry and orientation during cell division [23]. Unlike the randomly oriented cleavage plane of dividing cells in unstimulated cells, ES causes preferential alignment most often around 90° to the field vector [110].

A different hypothesis based on alterations in transmembrane potential is also described to justify the enhanced proliferative outcome of cells exposed to EF in culture. The first correlation between TMP and proliferative ability came from observations that non-dividing cells possess a very high TMP, whereas highly proliferating cells have low TMP [111]. Thus, the differential response of cancer

cells and stem cells to external EF can be understood based on their bioelectric properties. The electrophysiology of many cancer cells reveals them to possess depolarized membrane potential, that confers high cell proliferation. On the contrary, hyperpolarization is a pre-requisite for stem cell differentiation, wherein osteogenesis and adipogenesis of hMSCs were inhibited under depolarizing conditions [112]. The terminally differentiated cells would respond to EF by virtue of their characteristic membrane potentials. In our previous works, we ascertained that EF stimulation and substrate conductivity of the culture platform induced proliferation arrest and premature differentiation of C2C12 mouse myoblasts [113], neuroblastoma and schwann cells [52], fibroblasts and osteoblasts [81] as well as hMSCs [76].

Based on the assumption of nonlinear characteristics between ionic currents and TMP, exposure to EFs can either raise the TMP of dividing cells or lower the high TMP in quiescent cells, thus enhancing or reducing proliferation (Fig. 3)[114]. Alongside, studies also confirm other important molecular determinants sensitive to alterations in membrane potential, such as voltage active K^+ channels as well as gap junctions. These channels counter variations in membrane potential by shifting their voltage sensors across the electric field between the inner cytoplasmic and outer extracellular electrolytic solutions [115], [116]. While gap junction expression is necessary to maintain proliferation in adult stem/progenitor cells during regeneration [117], K^+ channels have been implicated as important players in regulating cell-cycle progression [118]. Another mechanism that is likely to induce cell proliferation via electrical stimulation (ES) is through a signaling pathway that involves calcium/calmodulin, nitric oxide synthase, nitric oxide, and cGMP [119]. ES modulates binding kinetics of Ca^{2+} calmodulin (CaM) by a twofold increase [35]. Ca^{2+}- CaM catalyzes the release of NO by the action of Nitric oxide synthase (NOS), which can subsequently activate cGMP that enhances growth factor activity to induce cell proliferation (Fig. 4) [120]. A different hypothesis points towards the release of pre-synthesized growth factors following EF exposure. The accumulation of these secreted growth factors in the culture medium could result in enhanced cell proliferation. As an example, the increased transcription of IGF-II mRNA and IGF-II secretion were recorded in cells, following the application of low-frequency electromagnetic fields [121].

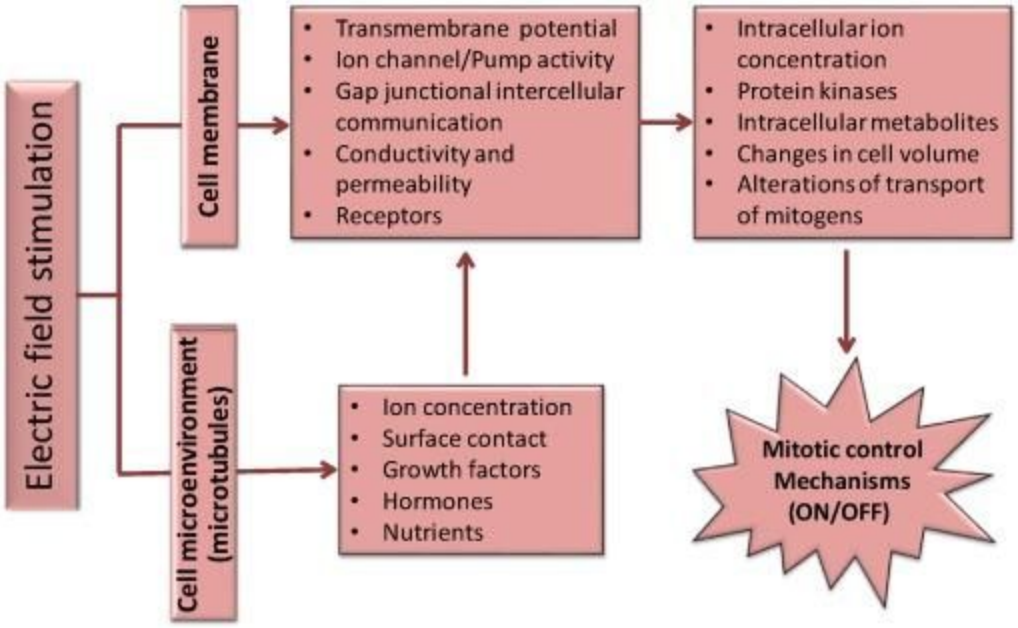

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Fig. 3. Possible ways by which EF controls mitosis in cells: Intracellular EFs generated by the synchronized oscillations of microtubules, centrosomes and chromosomes regulate mitosis. Exogenous EFs can affect the mitotic spindle assembly by interaction with its polar microtubules, that possess permanent electric dipole moment. A disruption of these cytoskeletal tubulin components by EF can affect the cellular microenvironment hindering ion transport, nutrients, proteins and small molecules essential for cell division. In parallel, at the cell membrane, EF can alter transmembrane potential, activity of voltage-gated ion channels, gap junctions and conformation of cell surface receptors leading to fluctuations in intracellular ion concentrations, enzymes, metabolites and mitogens. Cumulatively, EF can either induce cell proliferation or cell division arrest through the above interlinked mechanisms.

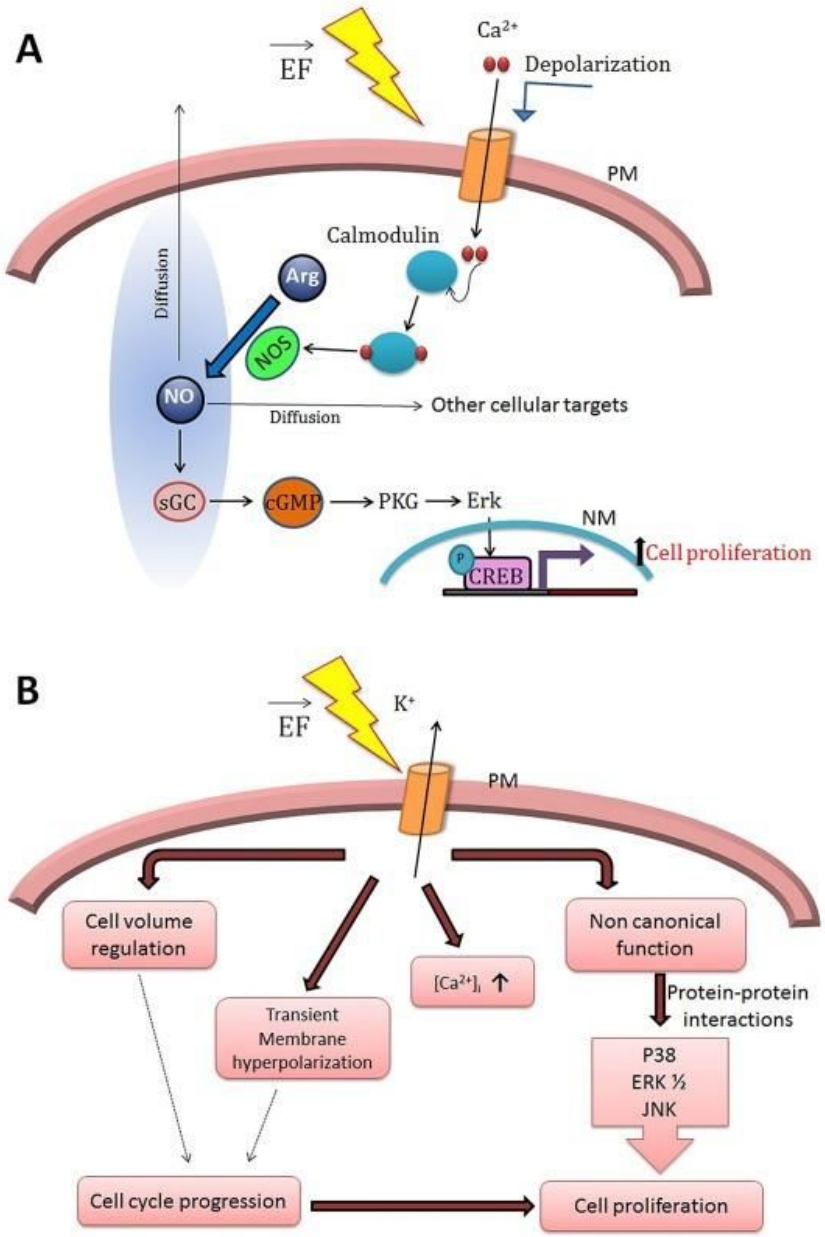

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Fig. 4. Schematic representation of proposed intracellular effects by which EF exerts its effect on stem cell proliferation. (A) One possible mechanism proposed involves enhanced cGMP activation by regulating calcium binding to calmodulin, resulting in the transient increase in NO production. (B)

Activation of K^+ ion channel is also considered as a potent molecular determinant involved in EF induced proliferative response. K^+ ion channels are known to influence cell cycle progression through K^+ flux, which leads to change in membrane potential, cell volume changes and increase in intracellular Ca^{2+}concentration. In addition, permeation-independent mechanism involving direct or indirect interaction between K^+ channels and proteins may also trigger proliferation [122], [123], [124]. PM: Plasma membrane, NM: Nuclear membrane, Arg: L-arginine, NO: nitric oxide, NOS: nitric oxide synthase, sGC: soluble guanylyl cyclase, cGMP: cyclic guanosine monophosphate, PKG: cGMP-dependent protein kinase, Erk: extracellular signal-regulated kinase, CREB: cAMP response element-binding protein.

3.2. Stem cell migration and orientation

During wound healing and tissue repair, the migration of local stem cells to the wound site is largely controlled by endogenous EFs. On this basis, manipulation using exogenous EF was widely pursued to guide stem cell migration, *in vitro* to achieve better wound healing and regeneration, *in vivo*[92]. Under an applied EF of physiologically relevant magnitude, some cancer cell types migrated towards the cathode, some to the anode and others showed a lack of any directional response [125]. Unfortunately, the migratory response of stem cells to EF cannot be ascertained from published literature, because EFs elicit significantly different cell motility responses, depending on the cell type as well as passage, time and voltage applied. For instance, hMSCs and human induced pluripotent stem cells (hiPSCs) were shown to migrate towards anode, while hESCs (human embryonic stem cells) and EpSCs (epithelial stem cells) migrated towards the cathodal side [126], [127], [128]. The application of DC EFs of physiologically relevant magnitude (30–250 mV/mm) directed NSC migration, demonstrating its effectiveness to guide neurite growth and electrotaxis of neurons and other cell types [129]. Selectively, DCEFs were capable of inducing galvanotaxis of adult NSCs towards the cathode, but not the differentiated populations [130]. A similar cathodal migration of NSC-derived oligodendrocyte precursor cells was observed in another study [131]. This voltage-dependent migratory activity was found to be mediated by one of the regulators of actin nucleation, i.e actin-related proteins 2 and 3 complex (ARP2/3). In another study, applied DC EF of 150 mV/mm guided cell migration of endothelial progenitors towards the cathode and such migration was mediated by VEGF receptor signaling, *in vitro*[132].
Recent resurgence of interest for human induced pluripotent stem cells (hiPSC) over ESCs, steered many investigators to probe into the effect of externally applied EF on hiPSC migration and differentiation [127]. A small EF could induce significant directional migration of hiPSC cells cultured both in 2D or 3D environment (Fig. 5C). Even cancer stem cells reacted to a weak DC EF stimulus, by moving towards the cathode with a distinct morphological change and directional response within 30 s [133]. The overexpression of EGF receptor, ErbB1 is hypothesized to trigger EF-guided migration of cells [125]. Furthermore, EF-induced asymmetric polymerization of F-actin and migratory orientation of rat EpSCs was deduced to be mediated via EGF receptors, ERK1/2 and PI3K/Akt signaling axis (Fig. 5B) [134]. DC EFs also caused murine ASCs to align at right angles to the field vector and elicited cathodal migration, that was field strength dependent. Additionally, their electrotactic response suggests the involvement of natural chemotactic signaling pathways that drive cell migration of multiple cell types [135]. Calcium signaling also plays a crucial role in the electrotaxis of cells [136]. Both intracellular calcium and extracellular calcium accumulation at the cathode during EF stimulation influence cell migration [137]. High DCEF intensity of 1400 mV/mm caused an elevation in intracellular calcium $[Ca]_i$ in both rat calvarial osteoblasts and human SaOS-2 cells by

involving voltage gated calcium channels (VGCCs), but with $[Ca]_i$ accumulation on the cathodal side in the calvarial osteoblasts and anodal side in SaOS-2, thus conferring differential directionality [138]. At higher extracellular calcium concentrations, the calcium ionic flow towards the cathode directed the migration of mouse fibroblasts towards the cathode in an EF strength dependent manner and the depletion of calcium by chelation with ethylene glycol tetraacetic acid (EGTA) led to a decrease in cathode-directed galvanotaxis [139]. A similar phenomenon was observed in the case of human keratinocytes stimulated with DCEF of 100 mV/mm, independent of extracellular calcium concentration [140]. On the contrary, low DCEF intensities of 50–300 mV/mm acted as a directional cue for the migration of bone marrow derived mesenchymal stem cells (BMSCs) to the cathode and macrophages towards the anode with electrotaxis regulated by EGFR accumulation and calcium, respectively [89].

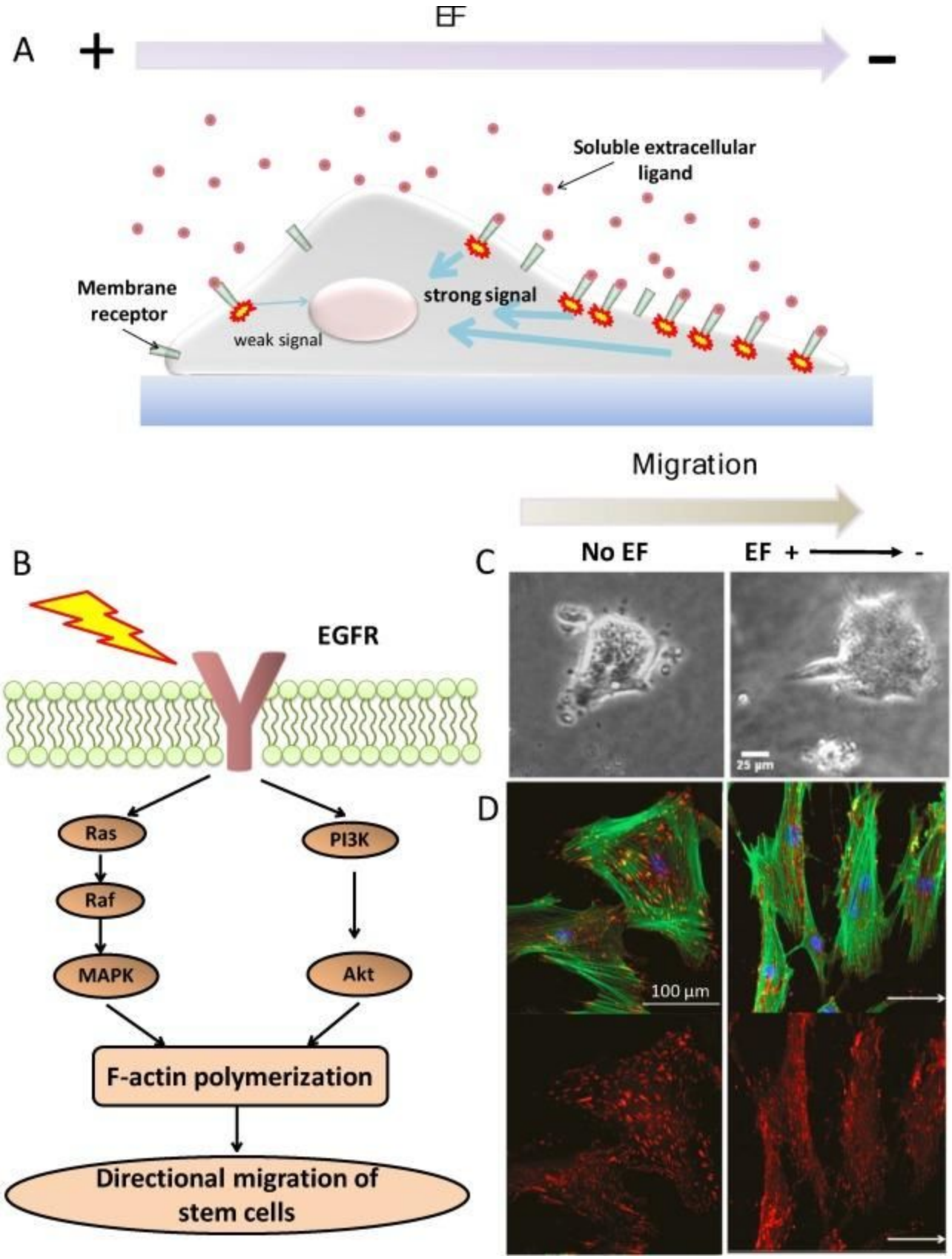

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Fig. 5. (A) EF-driven cell migration via asymmetric receptor redistribution. Exogenous EF induces the cell surface receptors to concentrate at the cathodal side, resulting in asymmetric amplification of cytoplasmic signaling at the leading edge of the cells. In some cases, exogenous EF can even

establish ligand gradient leading to asymmetric cytoplasmic signaling because of the increased availability of more ligands for the receptors on one side of the cell to become activated than on the other side. (B) Activation of EGFR signaling pathway by EF and its involvement in cell migration. The process of cell migration is mediated by the activation of Ras/Raf/MAPK and/or PI3K/Akt/mTOR pathway which is implicated in the organization of the actin cytoskeleton in the migratory cell. (C) Directed migration of hiPSCs by DC EF application, with a critical EF limit of <30 mV/mm (Reproduced with permission from Ref. [127]). (D) Cellular alignment of hMSCs in response to applied EF. (Reproduced with permission from Ref. [8]. Copyright © 2015, Royal Society of Chemistry). The cells oriented at 90° relative to the EF vector, with elongated morphology. Both actin stress fibres (green) and focal adhesion complex (red) aligned at right angles to the EF vector, for EF strengths in the range of 50 mV/mm to 300 mV/mm. Nuclei is stained blue.

While, exposure to DC EF has been proven to be effective in directional migration, the published results from clinical studies suggest the competence of pulsed EF in galvanotaxis [141]. In addition, balanced biphasic waveforms also can evoke a galvanotactic response in undifferentiated NPCs and has the added advantage of preventing charge accumulation, that is associated with DCEF. As a general rule, the field strength of applied EFs must be judiciously chosen to elicit cell migration without causing cell damage. Short exposure times can decrease the migration efficiency, while excessive exposure times can lead to cell death. With respect to cellular orientation, steady (DC) extracellular EFs have been shown to accelerate and orient the growth of embryonic neurites in cultures of explanted neurons. In addition to guiding neurite outgrowth, DCEFs could increase the length of the cell bodies, apart from modifying their orientation parallel to the direction of applied EF [27]. The preferential neurite growth or bending toward the cathode was found to be due to the field-induced cathodal accumulation of membrane glycoproteins, especially concanavalin A receptors by the process of passive electrophoretic receptor clustering on the cell surface under an external EF [142]. Both human adipose tissue-derived stem cells (hASCs) and human epicardial fat-derived stem cells (heASCs) were observed to elongate and align in the presence of DC EF of strength 600 mV/mm [143]. Conversely, rat MSCs reconstituted in 3D collagen scaffold failed to align due to their stronger 3D adhesion that resisted cell reorientation [144]. The minimum DC current density required to reorient the growth direction at the growth cone within 15 min was 0.2–2 pA/μm^2 (or 0.3–3 mV/mm). In the case of focal pulsed currents (pulse duration 5 ms), a typical combination of threshold pulse amplitude and frequency of 4 pA/μm^2 and 10 Hz, respectively were required [145]. A theory suggests that the cells tend to elongate and to align their cytoskeletal major axis perpendicular to the force direction to minimize the EF gradient (Fig. 5D) [146]. To sum up, EF guided motility, elongation and orientation of stem cells can be utilized as a promising approach for future therapeutic strategies with the limitation of engineering adult stem cell populations in therapeutically relevant numbers within implanted grafts or direct the homing of stem cells in sufficient numbers toward sites of injury to elicit wound repair.

3.3. Stem cell lineage commitment

In a manner identical to the electric field guided morphogenetic development of the embryo to generate different tissues, exogenous electrical stimulation can be applied for the lineage commitment of stem cells. Specific EF parameters can direct the stem cell lineage, based on the inherent electrical conductivities and electrical signals of the body tissues. Table 2presents a range of electrical conductivities for different mammalian tissues, which are of the order of ~10^{-3} S/cm for the heart, brain and muscle tissue, while it ranges to ~10^{-4} S/cm in the case of cortical bone and 10^{-5} -

10^{-6} S/cm for wet to dry skin. These values vary with the tissue source, measurement mode and their inherent anisotropic tissue organization. Also, electrical signals vary with injury as well as the wound location. For example, skin wounds generate endogenous EF of 100–200 mV/mm, while corneal wounds are around 40 mV/mm [147]. In the light of the variation in electrical conductivities and electrical signals in different tissues, it is possible to modulate stem cell differentiation towards particular lineage commitments by varying the EF stimulation conditions. The following sub-sections describe the lineage commitment of stem cells guided by external EF.

Table 2. Electrical properties of mammalian tissues, *in vivo*.

Tissue type	Electrical conductivity (S cm^{-1}) at ~50–100 Hz	Reference
Brain	(2.5–3.0) x 10^{-3}	Gabriel et al., 2009 [148]
Heart	5×10^{-3}	Gabriel et al., 2009
Skeletal muscles (Anisotropic)	(1.5–2.0) x 10^{-3}	Gabriel et al., 2009
Wet bone (Anisotropic)	Cancellous: (1.6–2.0) x 10^{-3}; Cortical: (5.8–6.3) x 10^{-4}	Saha et al., 2009 [149]
Skin	10^{-5} – 10^{-6}	Gabriel et al., 2009

3.3.1. Osteogenic differentiation

The concept of using small electric currents for inducing osteogenic differentiation of stem cells was prompted mainly by the discovery of piezoelectric properties of bone [150], [151]. Despite numerous evidences showing various forms of EFs in enhancing osteogenic commitment, a direct comparison of these results is not possible due to variations in the type of current applied (i.e direct, electromagnetic or pulsed), and other parameters such as amplitude, duration, frequency, stimulation mode, electrode material, etc. For instance, it was elucidated that a 50 Hz pulsed electromagnetic field was the most potent effector of osteoblast differentiation in hMSCs amongst a range of frequencies tested, including 5, 25, 75, 100, and 150 Hz [152]. Altogether, the widespread and increasing application of EF in clinical, *in vitro* and *in vivo* studies exemplify its tremendous potential as a clinically useful adjunctive intervention in regenerative therapies.

Hronik-Tupaj et al. [153], conducted the electric stimulation studies on hMSCs by exposing the culture system to an EF of 2 mV/mm, 60 kHz for 40 min daily. A delayed increase in osteogenic gene marker expression (ALP and Collagen I) was detected within 10 days in EF treated hMSCs, when compared to non-stimulated controls treated with osteogenic chemical inducers [153]. In stark contrast to this study, the application of high frequency capacitive electric field (2 mV/mm, 60 KHz) led to chondrogenesis in ASCs, with more expression of type II collagen and less type I & X collagen [154]. Other studies even indicate that PEMF alone is not sufficient to provide osteogenic stimuli to MSCs. Instead, culturing on ECM-like environment in the presence of soluble bio-factors, act together with EF stimulation to induce osteodifferentiation in hMSCs [155]. In another study, MSCs embedded inside collagen hydrogel constructs, when treated with alternating electric current in the absence of exogenous biochemical factors, induced osteogenic differentiation [156]. A large percentage of hMSCs underwent osteogenic differentiation under PEMF exposure with almost 20–60% higher cell densities in cultures at the exponential stage of expansion [157]. Quite a number of other reports also revealed enhanced levels of alkaline phosphatase (ALP) activity and mineral deposition in both adipose and bone marrow derived MSCs, under EF stimulation [158], [159]. Notably, EF exposure modulates the mRNA expression of certain bone marker genes to promote stem cell osteogenesis during various stages of osteogenic differentiation process [160]. For example, pulsed EF exposure resulted in the accelerated upregulation followed by downregulation

of cbfa1 transcripts during osteogenesis. On the other hand, in the case of bone morphogenetic protein 2 (BMP-2), mRNA expression level was initially low followed by a late upregulation of BMP2 transcripts [161]. It has been demonstrated that hMSCs exhibit elevated intracellular Ca^{2+} levels in response to electromagnetic stimulation, thereby serving as a key signal during osteogenic differentiation [162].

3.3.2. Neurogenic differentiation

The notion about the limited capability of brain to regenerate was shattered as they identified neural stem cells (NSCs) in anterior sub-ventricular zone (SVZ) of the forebrain and hippocampus. However, only limited populations of the newly generated NSCs are ultimately recruited to the sites of injury, and undergo consequential differentiation into the necessary neural cell types [163]. Knowing the involvement of endogenous EFs in neurogenesis, axon guidance, and nerve growth, several groups adopted the biophysical approach of EF stimulation to regulate the migration and integration of NSCs in the mammalian CNS [164]. A very high gene expression of maturation markers, ENO2 and MECP2 was apparent at an early culture stage in electrically (5 mV, 0.5 mA, 25 ms intermittent stimulation) stimulated neural stem cells (NSCs) cultured on conducting ropes, which is a hallmark of neuronal differentiation of NSCs [165]. In another study, apart from inducing directional migration of NSCs, DC EF stimuli of 115 V/m field strength could also enhance differentiation specifically into neurons, but not to astrocytes or oligodendrocytes [129]. Although questionable, the exposure of NSCs to oscillating AC EF of 1 Hz induced a predominant differentiation into astrocytes over neurons [166]. Other examples include electro-stimulation with parameters of 4–8 $\mu A/cm^2$ current density, pulse duration of 200 µs and frequency of 100 Hz caused the largest increase in cell multiplication and neural differentiation of fetal NSCs [108]. Unlike non-stimulated embryoid bodies (EBs), EF stimulated EBs assumed neuronal fate by adopting the appearance of various types of neuronal cells, when injected into injured spinal cord, an environment that is non-neurogenic [167].

DC stimulation is the most widely studied among the EF stimulation modalities to date, for remedial neural regeneration and neurite outgrowth through an injured or ischemic site [168]. On the other hand, it was validated that AC stimulation with high duty-cycle (1-kHz 80% duty-cycle) is also capable of eliciting a neural response similar to DC ES, with additional benefits of higher field propagation and lower consumption of electrical power. When NSCs are exposed to electromagnetic stimulation, spontaneous Ca^{2+} transients are developed due to increased Ca_v1-channel (L-type) activity [169]. Ca^{2+} influx through Ca_v1 channels stimulate neurogenesis by Ca^{2+}-related signal transduction pathways, that are mainly mediated by phosphorylation of CREB transcription factor. Especially, in the case of EF stimulated bone marrow derived hMSCs, a rise in neuronal marker gene expression level was determined to be mediated by CREB phosphorylation [170]. It is also speculated that EF exposure increases CREB DNA binding activity, particularly to a class of polynucleotidesknown as cAMP [171]. Such a process is crucial for a number of cellular phenomena including cell proliferation, differentiation, and other adaptive functions [172]. Challenging this prevailing claim of L-type channel activity by EF, Brosenitsch and Katz [173]demonstrated the pivotal role of N-type calcium channels in neuronal gene expression. Notwithstanding, neural cells can sense differences in the calcium entry from two distinct subtypes of voltage-activated calcium channels and can accordingly recruit specific intracellular signaling pathways to trigger subsequent gene expression.

3.3.3. Cardiomyogenic differentiation

The cardiac muscle tissue in the native heart has specialized electrical signal conduction and propagation pathways such as electromechanical coupling via gap junction channels and this coordinated electrical signaling mechanism is involved in heart contraction [51], [174]. Ideally, the creation of a cardiac-mimetic electrical environment during *in vitro* culture should result in the progressive generation of functional cardiac tissue. Yet, biologists toy with traditional static cell culture methods to maintain differentiated cardiac cells, despite its greater tendency to dedifferentiate, *in vitro*[175]. But the scenario reversed eventually with the advent of cardiac tissue engineering, wherein a combination of mechanical, electrical, and functional integration was realized as an ideal approach to generate engineered cardiac constructs [97]. A detailed genome microarray analysis of ESCs after electrical pacing revealed an apparent decline in the expression of genes governing self-renewal and pluripotency such as Oct4 and Fox3. Simultaneously, a number of genes and pathways related to early mesodermal, cardiac and neural development and differentiation were significantly upregulated [176]. Previous works have also elucidated that EF stimulation promotes the expression of both early and late cardiac-specific genes in hESC-derived cardiomyocytes [177]. Further, an increasing number of publications showed the association of exogenous EF in inducing cardiogenesis in embryoid bodies (EBs) derived from hESCs [4], [178]. Despite the fact that hESCs can differentiate into cardiomyocytes via EB formation, the low percentage of differentiated cardiomyocytes and heterogeneity of cell populations limit the usage of hESC derived cardiomyocytes for clinical applications [179], [180]. A brief electrical stimulation (65 mV/mm or 200 mV/mm field strength, 1 Hz frequency, 1 ms pulse width) for 2 weeks after cell plating led to a marked escalation in the fraction of beating EBs along with pronounced augmentation in cardiac gene expression level (Fig. 6B). In addition to the stem cell plasticity related transdifferentiation ability of MSCs to cardiomyogenic progenitors post-transplantation in the myocardium [181], pulsed electrical stimulation either in the presence of conducting nanostructures [182] or demethylation agent 5-azacytidine [183] or a combination of both [76] is validated to promote cardioprogenitor phenotype in MSCs (Fig. 6A). Likewise, stimulation with monophasic square-wave pulses of 5 mV/mm was proven to be effective in evoking cardiomyogenic differentiation potential even in adipose tissue-derived progenitor cells in 2D and 3D culture milieu [184], [185]. Here, it may be important to emphasize the controversy around the transdifferentiation of MSCs into functional cardiomyocytes. Most literature reports indicate that bone marrow derived MSCs can be induced to express cardiomyogenic marker proteins upon co-culture with cardiomyocytes *in vitro*, but they do not develop into contractile electrically active functional cardiomyocytes [186], [187], [188]. However, the authors believe that electrical stimulation can be used optimally to such co-cultures along with chemical inducers to differentiate MSCs into beating cardiomyocytes. It must be noted without any conclusive experimental evidences, the transdifferentiation of MSCs would remain highly controversial.

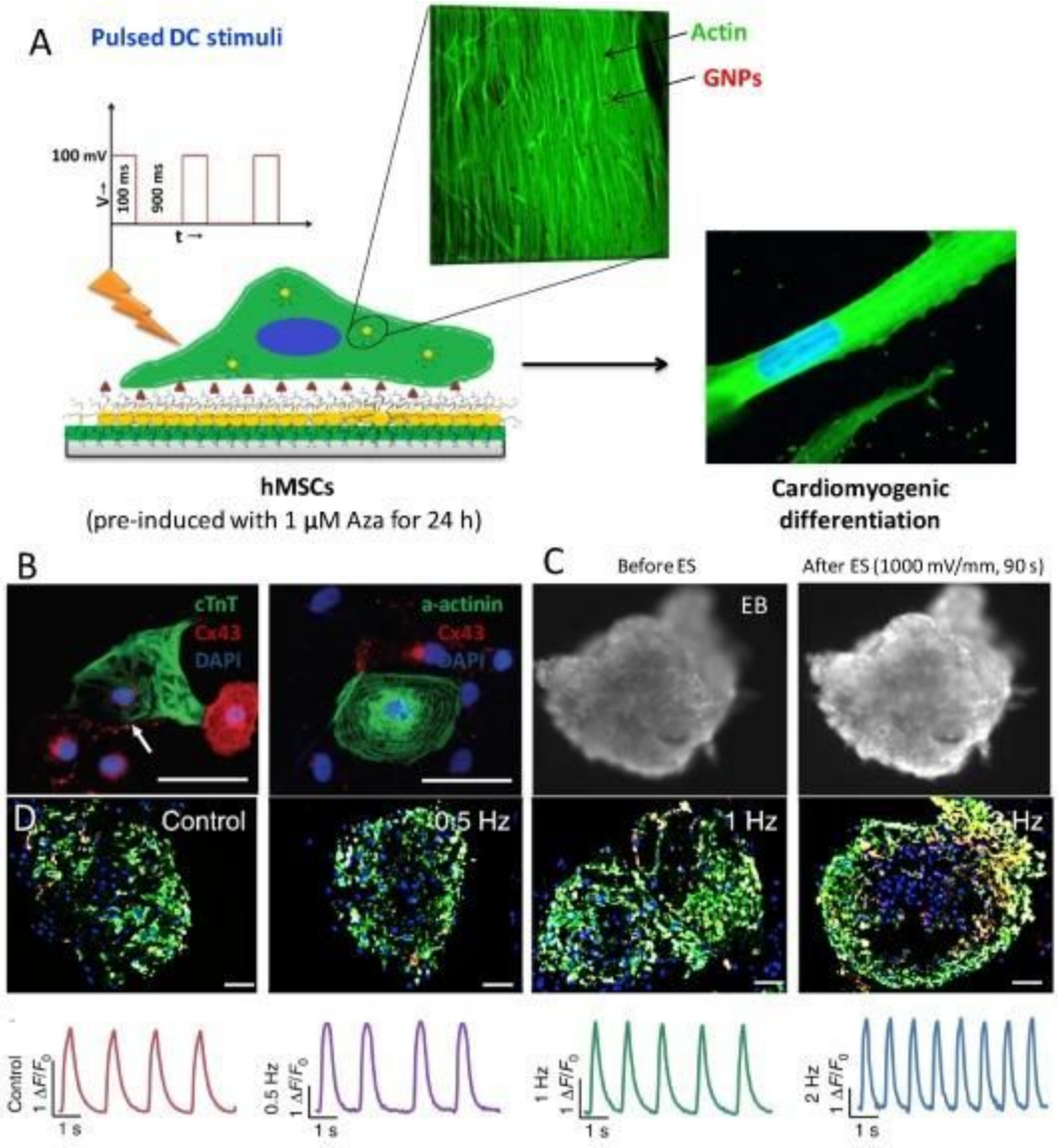

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Fig. 6. Electrical stimulation induced cardiac differentiation of stem cells. (A) hMSC differentiation induced by electric field assisted gold nanoparticles (GNP) actuation [76]. Fluorescence image in the right top corner shows the tube-like morphology exhibited by intra/extracellularly electroactuated hMSCs at 1 Hz frequency (cytoskeletal actin - green and nuclei - blue). (B) Expression of cardiac-specific markers in hiPSC derived cardiomyocytes that were electrically stimulated at 200 mV/mm for 5 min. Scale bar: 50 μm (Reproduced with permission from Ref. [180], under Creative Commons Attribution 4.0 International Public License: http://creativecommons.org/licenses/by/4.0/). (C) Increased ROS production in embryoid bodies (EB) after EF application at 1 V/mm for 90 s. ES enhanced the number of cardiac-differentiated hESCs by the downstream activation of ROS-triggered signaling pathway. Fluorescence micrograph of EB loaded with dichlorofluorescein (DCF) before and 20 min after ES (90 s) is shown in the image. (Reproduced from Ref. [4], Copyright 2009, with permission from Elsevier). (D) hESC derived cardiomyocytes adapting their natural beating frequency to the EF stimulation frequency, an effect mediated by the expression of troponin (green) and hERG potassium channels (red). Cells were counterstained with DAPI (blue nuclei). Scale bar: 50 μm. Frequency dependent change in calciumcycling upon ES is shown in the bottom panel. Calcium imaging on spontaneously beating EBs was performed after 7

days of ES, after loading the cells with a calcium sensitive dye (Fluo-4 AM). Adopted with permission from Ref. [192], under Creative Commons Attribution 4.0 International Public License: http://creativecommons.org/licenses/by/4.0/).

Almost all the ES studies performed to date for cardio-differentiation involves the use of 1–2 Hz square-wave monophasic pulses, rather than the use of a steady current. These pulsed signals are implicated in the fusion of mononuclear cells to form a multi-nucleated cardiac symplasm or syncytium. This is in contrast to the DC signals that are involved in direct cell migration and morphological changes during the developmental stage [60]. Correspondingly, several investigations confirmed enhanced beating of cardiomyocytes [189], myotube contractile behavior and sarcomeric assembly [190] upon stimulation with low frequency electric pulses. Particularly, electrical stimulation with frequency of 1–2 Hz activated transient surges in intracellular Ca^{2+} levels along with the augmented sarcomere assembly and maturation, while higher frequency (10 Hz) failed to trigger such effects in myotubes [191]. With respect to pulse duration, a time period of 1–2 ms is ample time to excite cardiac tissue and cells, and besides, this signal span is adequate to collapse the double layers formed on the stimulation electrodes between successive pulses.
From a tissue engineering perspective, Tandon et al. [60] elegantly described the protocol to deliver pulsatile electrical fields to engineered 3D constructs, mimicking cardiac tissue. Electrical conditioning of hESC-derived cardiomyocytes in such 3D aggregates promoted the generation of mature, electromechanically coupled cells (Fig. 6D). It was also found that the stimulation frequency alone can promote the final and complete functional maturation of cardiomyocytes [191]. Further exploratory studies also revealed that the stimulatory effect of EF on cardiomyogenesis of hESCs is also routed through mechanisms involving intracellular ROS production (Fig. 6C) [4]. Recently, efforts have also been made towards the development of electrical stimulation bioreactors to examine the roles of multiple ES parameters, such as the nature of the electrode material, period of electrical stimulation and developmental/maturation stage of the cells in a single microfluidic device [4].

3.3.4. Angiogenesis

In line with the myriad of cell responses, such as cytoskeleton rearrangements, migration, proliferation, and differentiation, exogenous EF exposure is also known to induce angiogenesis [193]. The endogenously occurring differences in bioelectric potentials are present in and around the blood vasculature, both in relation to fluid flow of blood and in locations of neovascularization, such as during development of the fetus and cancer metastasis [194]. In addition, EF can initiate and guide blood vasculature formation by activating the VEGF receptor signaling pathway, to evoke important pre-angiogenic cellular events, *in vivo.* Apparently, endothelial cells respond to EF by an increased secretion of VEGF and subsequent activation of VEGF receptors (VEGFRs), phosphatidylinositol-3-kinase (PI3K)-Akt and Rho-ROCK elements of the VEGFR signaling pathway [194]. Even in osteoblasts, biphasic electric current induced a 3 fold enhancement in the secretion of VEGF [195]. Also, hASCs exposed to DC EF of 600 mV/mm for 2–4 h upregulated the gene expression of connexin-43, thrombomodulin, VEGF, and FGF [143]. Electrical exposure of EBs derived from mouse ES, resulted in robust expression of HIF-1α and VEGF, followed by

endothelial differentiation and vasculogenesis. It was also deduced that the stimulation of angiogenesis in ES was mainly mediated by the activation of ERK1,2 and JNK, whereas p38 MAPK activity was non-essential [19]. Interestingly, the pro-angiogenic response due to high frequency EF stimulation does not require the direct binding of VEGF to VEGFR2 receptor, rather it occurs via a frequency-sensitive VEGFR2-independent activation of the MAPK/ERK pathway [196]. Even, pulsatile ES of low strength could cause a marked increase in vascular permeability, uniform blood flow and vasculature density in the ischemic limb of rats by augmenting the *de novo* synthesis of VEGF protein [197], [198]. Nonetheless, persistent ES in the skeletal muscle tissue might be unsafe, as it fails to reinstate blood flow and promotes muscular atrophy as well as worsened fatigue [198]. All these results reinforce the idea of applying ES to trigger cells in the ischemic tissue zones to bio-manufacture required amounts of endogenous VEGF proteins, so as to promote local angiogenesis. Such strategies offer new perspectives to repair post-stroke neural damage or ischemic myocardial damage by combining stem cells with external ES as a multifaceted approach for neovascularization.

Summarizing, the electric field induced stem cell response in terms of proliferation, migration and differentiation to osteogenic, neurogenic and cardiomyogenic as well as angiogenesis under particular EF stimulation parameters and protocols have been described so far. Table 3 presents an overview of EF stimulation effects on stem cells, *in vitro*. A glance at the electrical stimulation parameters in Table 3 will reveal EF intensities of the order of 10–100 mV/mm with pulse durations of 10–100 ms and low frequencies of 0–2 Hz to be optimal for effective proliferation and differentiation of lineage committed stem cells. However, for the electromechanical coupling of cardiomyocytes leading to contractile activity and electrical signatures, high EF intensitites of 300–500 mV/mm with short pulse widths of 1–2 ms and frequencies close to that of the native heart rate (1.2 Hz) and accelerated heart rate (2–3 Hz) are applied.

Table 3. An overview of EF stimulation effects on stem cells *in vitro*.

Electrical stimulation	Stimulation parameters	Cell type	Outcome of the study	Reference
Capacitative-resistive electric transfer (CRET)	448 kHz, 50 μA/mm^2, 5 min 'On' per 4 h for 48 h	Adipose derived stem cells (ADSCs)	Enhanced ADSC proliferation without any effect on their adipogenic, chondrogenic and osteogenic differentiation	Hernandez-Bule et al., 2014 [99]
Direct current (DC), Capacitive coupling (CC), pulsed electromagnetic field (PEMF), and Degenerate wave (DW)	10 mV/mm at 3 h/day for 5 days	Bone marrow mesenchymal stem cells (BMMSCs)	DW and CC promoted the expression of migratory genes and proliferation in BMMSC, while reducing apoptotic and cytotoxic effects	Griffin et al., 2011 [105]
Transformer-like coupling (TLC)	0.36 mV/mm, 7 ms, 10 Hz for 28 days	Human mesenchymal stem cells (hMSCs)	TLC generated a pulse electric field that promoted osteogenesis of hMSCs cultured on artificial ECM coated polyaniline substrates	Thrivikraman et al., 2015 [78]
Direct current electric field (DC EF)	16 mV/mm	Human neural stem cells (hNSCs)	Small DC EF of 16 mV/mm caused galvanotaxis of hNSCs	Feng et al., 2012 [126]

			and neurite outgrowth towards the cathode, while higher field strengths reduced the hNSC galvanotactic response	
Direct current electric field (DC EF) in a 6 well plate configuration	100 mV/mm at 1 h/day for 7 days	ADSCs from Sprague-Dawley rats	Osteogenic differentiation of ADSCs exposed to DC EF occurred with the upregulation of Runx2, osteopontin and osteonectin	Mobini et al., 2016 [40]
Direct current (DC EF) and pulsed electric fields (PEF)	10 mV/mm, 100 ms, f = 0.1, 1 and 10 Hz at 15 min/day	Human mesenchymal stem cells (hMSCs)	DC EF elicited neurogenic expression, while PEF of 1 Hz induced the upregulation of cardiomyogenic genes in hMSCs	Thrivikraman et al., 2016 [76]
Biphasic rectangular pulsed electric field	1-6 Hz, 1 ms, 3–4 mV/mm for 7 days	Cardiomyocytes derived from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs)	3D aligned cardiac tissues (biowires) with striations as well as electrophysiological activity and intracellular calcium oscillations were recorded.	Nunes et al., 2013 [94]
Biphasic voltage pulses using a commercial Myopacer (IonOptix)	10 V, 10 ms, 1 and 2 Hz	Cardiomyocytes derived from embryoid bodies (EBs) of hESCs	EBs encapsulated in 3D hydrogel networks exhibited spontaneous contraction behavior and responded to external electrical pacing	Chung et al., 2012 [42]
Square wave pulse stimulation (Grass Technologies)	500 mV/mm, 2 ms, 0.5, 1, 2 Hz	3D culture of Cardiomyocytes derived from EBs of hESCs and iPSCs	Electrical conditioning elicited maturation of cardiomyocytes, connexin expression and beating rate adaptability to stimulation frequency	Eng et al., 2016 [192]
Contactless EF stimulation via interdigitated array of Pt electrodes	10 V, 10 ms, 1 Hz for 2 days after 1 week culture	C2C12 mouse myoblasts cultured in microgrooves	EF stimulation promoted formation of mature skeletal muscle tissue constructs with contractile activity	Ahadian et al., 2013 [199]

4. Mechanisms of EF induced stem cell response

Several cellular effects are understood to be mediated by exogenous EF through a mechanism called electrocoupling. The basis of invoking such an indirect effect emerges from the high resistance imparted by the plasma membrane, which prevents the penetration of electric stimuli, regardless of the conducting nature of cytoplasm [18]. One of the possible electrocoupling mechanisms involves asymmetric redistribution/diffusion of electrically charged cell-surface receptors in response to electric field, which further activates numerous downstream signaling cascades. Another possible mode is directly via the activation of voltage-gated Ca^{2+} channels by cell membrane depolarization, that leads to the most consistently occurring cellular response to electric stimuli, i.e. the elevation in intracellular calcium ion concentration. A detailed explanation along with numerous hypotheses regarding the possible interaction mechanisms between EF and biological systems are discussed in the subsequent sections.

4.1. Signal transduction pathway

Electrical signals are sensed and converted into biochemical cues by multiple pathways within the cells, resulting in various biological responses. The activation of signal transduction pathways is considered as the possible mechanism by which the applied electrical stimulation could exert control over cellular functions. A major signal transduction pathway, which governs the transcription of specific mRNAs in response to external stimulation is the activation of the MAPK (mitogen-activated protein kinase) cascades.

The MAPKs are generalized family of serine/threonine kinases, that regulate intracellular events in response to extracellular signals [200]. The sequential activation of protein kinases within these cascades (extracellular signal-regulated kinases: ERK1/2 and ERK5, Jun amino-terminal kinases: JNK, p38MAPK) mediates numerous important cytological activities, including proliferation, differentiation, metabolism, cell cycle progression and apoptosis, depending on the type of stimuli and cell [201], [202]. Several literature reports raise the possibility of the activation of MAPK pathways in response to EF, although it remains to be determined methodically in every aspect. The EF induced elevated MAPK activation was documented in endothelial angiogenic response as well as in HL-60 (human promyelocytic leukemia cell line) differentiation [11], [196]. Mechanistically, cell motility and wound healing responses elicited by electrical field gradients occur through the dynamic regulation of PI(3)Kγ (phosphoinositide 3-kinase) and PTEN (phosphate and tensin homolog) signaling. An accelerated and progressive enhancement in the phosphorylation of extracellular-signal-regulated kinase (ERK), p38 mitogen-activated kinase (MAPK), Src and Akt on Ser 473 site was distinctly observed in cells undergoing electrotaxis [203]. It was also reported that the applied low-intensity 0.1 ms electrical current could induce a transient and low level activation of p38-p53 pathway, which is implicated to play significant roles in annihilation of malignant tumors as well as downregulation of inflammatory cytokine responses and metabolism [204]. The cellular orientation and movement of adult stromal cells in response to EF was observed to be linked to the activation of PI3K and ROCK signaling pathways [144].

It is also well-established that the gene expression of specific markers in lineage committed stem cells is mainly through the sequential activation of MAPK pathway centered around regulators of alpha-foetoproteins (Raf), ERK, p38 or jun-kinase 1/2/3, leading to the phosphorylation of transcription factors, which controls genotypic expression [205]. Earlier studies suggest c-Jun as the prominent downstream nuclear effector of MAPK/ERK pathway. c-Jun can form dimers either with itself or with other related transcription factors, like c-Fos. This is expected to result in the transcription of several AP-1 responsive genes that are shown to induce differentiation in stem cells [206].

The central pathway of signal transduction could be due to calcium influx through voltage-regulated calcium channels causing rapid phosphorylation of Src tyrosine kinases. This in turn leads to the formation of a Shc-Grb2 complex and Ras activation along with subsequent induction of downstream MAPK pathway [207]. In addition, Rho family members can also activate MAPK signaling pathways, so these small GTP (Guanosine-5’-triphosphate) binding proteins function as dual regulators of cytoskeletal remodeling and gene expression [125]. Moreover, membrane depolarization can

induce phosphorylation of tyrosine moieties in EGF (epidermal growth factor) receptors to an extent required to activate the ERK/MAP kinase pathway [208].

Alternate branch point of MAP kinase activation can be possibly coupled with the integrin, one of the major receptor that creates focal contact between cell and matrix. Accordingly, it can be postulated that in response to adhesive interaction to electroconducting substrates, the clustering or redistribution of integrin might induce the autophosphorylation of FAK (Focal adhesion kinase). FAK further complexes with Src, leading to Ras activation, while Ras in turn couples integrins to activate the ERK/MAPK signaling pathway [209]. Thus, depolarization induced activation of MAPK pathway links electrical cues to the alteration in gene expression and lineage commitment, a process analogous to those signaling cascades activated by the binding of growth factors to their specific CSRs (Cell surface receptors) [208].

Summarizing, cells respond to external electrical cues through complex and interconnected network of biophysical transduction signaling pathways modulating gene expression. Such phenomenon ultimately decides the fate of the cells. It is reported that the transcript levels for c-myc, c-fos, c-jun, and protein kinase C are elevated in multiple cell types treated with a sinusoidal EF, and under varied exposure conditions [210]. Likewise, the application of a specific and defined CC EF on osteoarthritic articular cartilage explants led to significant upregulation of collagen and proteoglycan genes and interleukin-induced downregulation of a number of metalloproteinases [211]. Previous studies demonstrate the blocking of signaling pathways inhibited EF induced proliferation, migration and differentiation of stem cells [212]. This suggests the mediation of stem cell response to external EF via signal transduction pathways. Overall, there is no doubt that the impact of EF on cellular functions can be attributed to enhanced signal transduction. However, aside from the above mentioned instances, several other intrinsic pathways that are specifically influenced by EF remain largely unexplored.

4.2. Ca^{2+} transients

One of the immediate effects in terms of cellular response to EF stimulation is the increase in intracellular Ca^{2+}. Calcium–mediated signaling regulates many different cellular processes and its impact is known to influence nearly every aspect of cellular life [213]. The alterations in free intracellular calcium concentration have been implicated in specific courses of cellular events in myriad cases, especially in the migration and differentiation of individual cells. The importance of intracellular calcium and extracellular accumulation at the cathode in electrotaxis has been briefly dealt in section 4.2. Here, the effect of calcium transients on stem cell differentiation is described. Intracellular Ca^{2+} increase is driven in two fundamental ways; by influx of Ca^{2+} from extracellular space via ion channels embedded in the plasma membrane or by the release of Ca^{2+} from the internal stores of endoplasmic reticulum (ER) via specialized receptor/channels on the ER [214].

The plasma membrane is the prime location of EF interaction with the cell, and it is hypothesized that an EF interference with membrane-mediated detection of biochemical signals, transduction and amplification may regulate many field guided phenomena in biological systems. Of note, EF exposure can directly stimulate L-type voltage gated Ca^{2+}channels (VGCCs) in the plasma membrane (Fig. 7) [215]. This can elicit many regulatory responses, including high levels of nitric oxide (NO) generated through the enzymatic action of the two Ca^{2+}/calmodulin-dependent nitric oxide synthases,

nNOS and eNOS [216]. In order to activate VGCCs, the applied EF must be adequately large to create an induced transmembrane potential difference of the order of 100 mV.

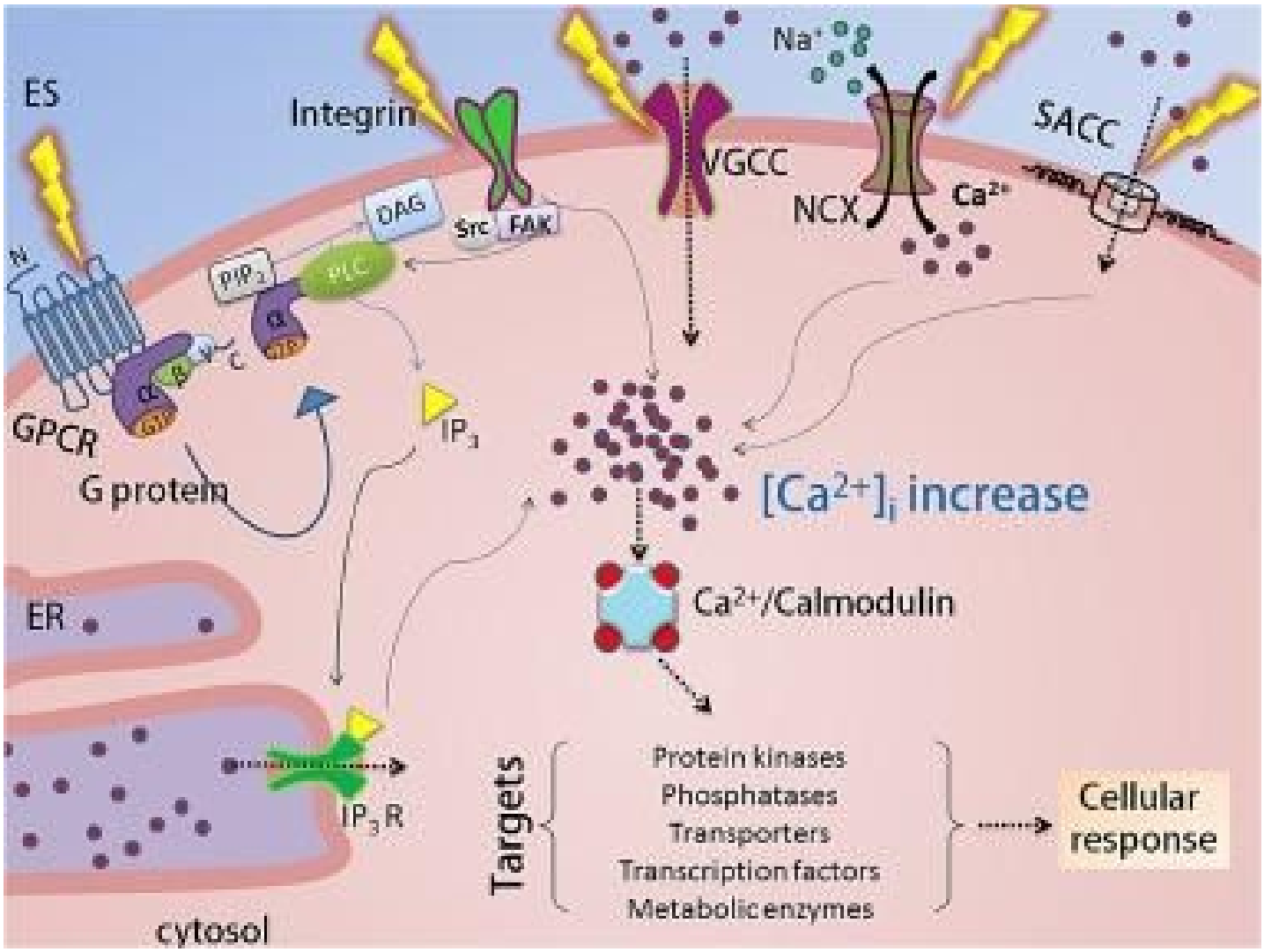

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Fig. 7. Schematic illustration of the possible pathways of calcium influx upon exogenous EF exposure. ES can elevate intracellular calcium level either through influx from extracellular pools through the activation of VGCC, NCX, SACC, etc. or through the release from endoplasmic reticulum stores via GPCRstimulation. EF can even trigger specific integrin subunits to regulate intracellular calcium currents via Src and FAK signaling.

Theoretically, voltage-gated ion channels are transmembrane helical sub-units containing several charged amino acids that can move in response to an applied EF. For these ion channels, the equilibrium between closed and open states varies within a small window of applied voltage [217]. Secondly, it is proposed that EF can stimulate stretch-activated Ca^{2+}channels (SACCs), which itself can mediate the spike in $[Ca^{2+}]_i$ by permitting the influx of Ca^{2+}[18]. A greater influx of Ca^{2+} from the extracellular space is routed through the Na^{+}-Ca^{2+}-exchanger (NCX). It is also speculated that ES can activate G-protein coupled receptors (GPCR), leading to phospholipase C (PLC) activity triggered synthesis of secondary chemical messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate present in the plasma

membrane (Fig. 7). Subsequently, the diffusion of IP_3 through the cytosol and binding to IP3 receptors (IP_3R) in the endo/sarcoplasmic reticulum, leads to Ca^{2+} release from internal stores [214], [218]. The GPCRs are highly sensitive to variations in the electrical properties of cell membrane and thus exogenous EF play a pivotal role in the energetics and dynamic conformational transitions of these receptors [219]. It also appears that several different integrins, activated by EF, appear to mediate calcium signaling via tyrosine kinase-dependent coupling and by other unidentified pathways that can couple integrins to calcium release both, from IP_3-sensitive intracellular stores, or to the influx of Ca^{2+} from extracellular space [220].

Ca^{2+} influx is necessary for the fate determination of stem cells and is implicated to play a central role in triggering calcium dependent cell signaling cascades at appropriate stages of stem cell differentiation [167], [221]. Especially, cells recognize even low intensity calcium signals, or transient calcium spikes, known as calcium oscillations through well-defined mechanisms to decipher cellular instructions encoded in the Ca^{2+} dynamics. Cells can then perceive amplitude or frequency modulation and discern the instruction encrypted in the calcium signal [221]. Notably, there is a proven correlation between the frequency of Ca^{2+}oscillations and the nature of the extracellular stimulus [222]. It is also compelling to note that the intracellular Ca^{2+} signaling cascades that are triggered in response to patterned ES are distinct from those arising from chronic membrane depolarization. This differential activation for specific intracellular protein kinases can differ based on the strength/amplitude of the cytosolic calcium signal [173]. Hence, the downstream effectors of signaling pathways can specifically recognize the source and spatio-temporal calcium profiles, thereby offering specific transcriptional activity.

Ca^{2+} concentration has been associated with EF-induced guidance of stem cell differentiation. The changes in $[Ca^{2+}]_i$ resulting from intrinsic cellular events or from programmed cell cascades, have been reported to prompt distinct series of cell behavior in many instances, like in osteogenic and neuronal differentiation of MSCs [221]. AC and pulsed electric fields induce an increase in $[Ca^{2+}]_i$ by promoting Ca^{2+} influx across the plasma membrane. For instance, the application of 1 or 10 Hz EF to human cells led to a 4-fold increase in $[Ca^{2+}]_i$ (from 50 nM to 200 nM) within 0.5 h of field exposure. Parenthetically, Ca^{2+} deficiency in the extracellular culture medium could not elicit the EF-induced elevation in $[Ca^{2+}]_i$, implying that extracellular Ca^{2+} influx across the plasma membrane is the reason for the observed $[Ca^{2+}]_i$ increase [18]. Indeed, this increase in $[Ca^{2+}]_i$ could be routed by either direct entry of Ca^{2+} ions through SACCs, or indirect entry through the NCX or VGCC, but the possible route has not been clarified.

Collectively, a link between electric cues, calcium entry and cellular response exist and the elucidation of the molecular mechanisms by which these second messengers act is outlined in Fig. 7. Besides that, a lot of efforts still need to be invested to seek a better understanding of the regulatory mechanisms governing EF-mediated Ca^{2+} increase. Overall, the effect of electrical cues on the stem cell behavior is a result of intricate interplay of events and pleiotropic effects involving Ca^{2+} ions, thereby mimicking and complementing the role of growth factors in mediating biological responses.

4.3. Mechanotransduction: cytoskeletal reorganization and actin distribution

Mechanotransduction is the conversion of external mechanical stimuli into intracellular electrical or chemical signals [223]. The consequence of mechanotransduction are manifested in the form of opening/closing of mechano-sensitive ion channels, changes in cell cytoskeleton or a cascade of biochemical signaling pathways governing self-renewal and differentiation in stem cells [224]. The inverse effect of mechanotransduction is the transformation of electrical stimulus into mechanical activity, that causes tension in the cytoskeleton due to reorganization of the cytoskeletal filaments and actin redistribution. Furthermore, electrical cues from extracellular space interacting with the plasma membrane also induce changes in the state of cellular actin [225].

In this regard, electric stimulation has been shown to extort either direct effects on the cytoskeleton, or on cellular processes mediated by the cytoskeleton [37]. For example, short electric pulses induced a field strength dependent polymerization of G-actin [225]. A transitory increase in the synthesis of cellular filamentous actin by 80% was recorded after ES, with an asymmetric distribution towards the cathode [226]. Similarly, direct evidence of EF induced disorganization of actin filaments were provided by Onuma and Hui [227]. Studies can also be found in the literature on the sensitivity of actin microfilament reorganization to the frequency of applied EF. Seemingly, the actin microfilaments were distorted and could not reorganize when exposed to rapidly oscillating electric fields [228]. The application of 200 mV/mm DCEF resulted in a near two-fold decline in the elasticity of hMSCs due to the disassembly of actin structures.

It was demonstrated that EF induced depletion of ATP led to the blockage of the ERM linker proteins that are known to bridge the plasma membrane and actin filaments, resulting in the partial disruption of cytoskeleton (Fig. 8D) [37]. Unlike the noticeable effect of DC EF on actin reorganization, microtubules or intermediate filament arrangement were found to be unaffected at the same field parameters tested. The Rho family of GTPases, including Cdc42, Rac and Rho, has been implicated in regulating the dynamics of actin microfilament [229].

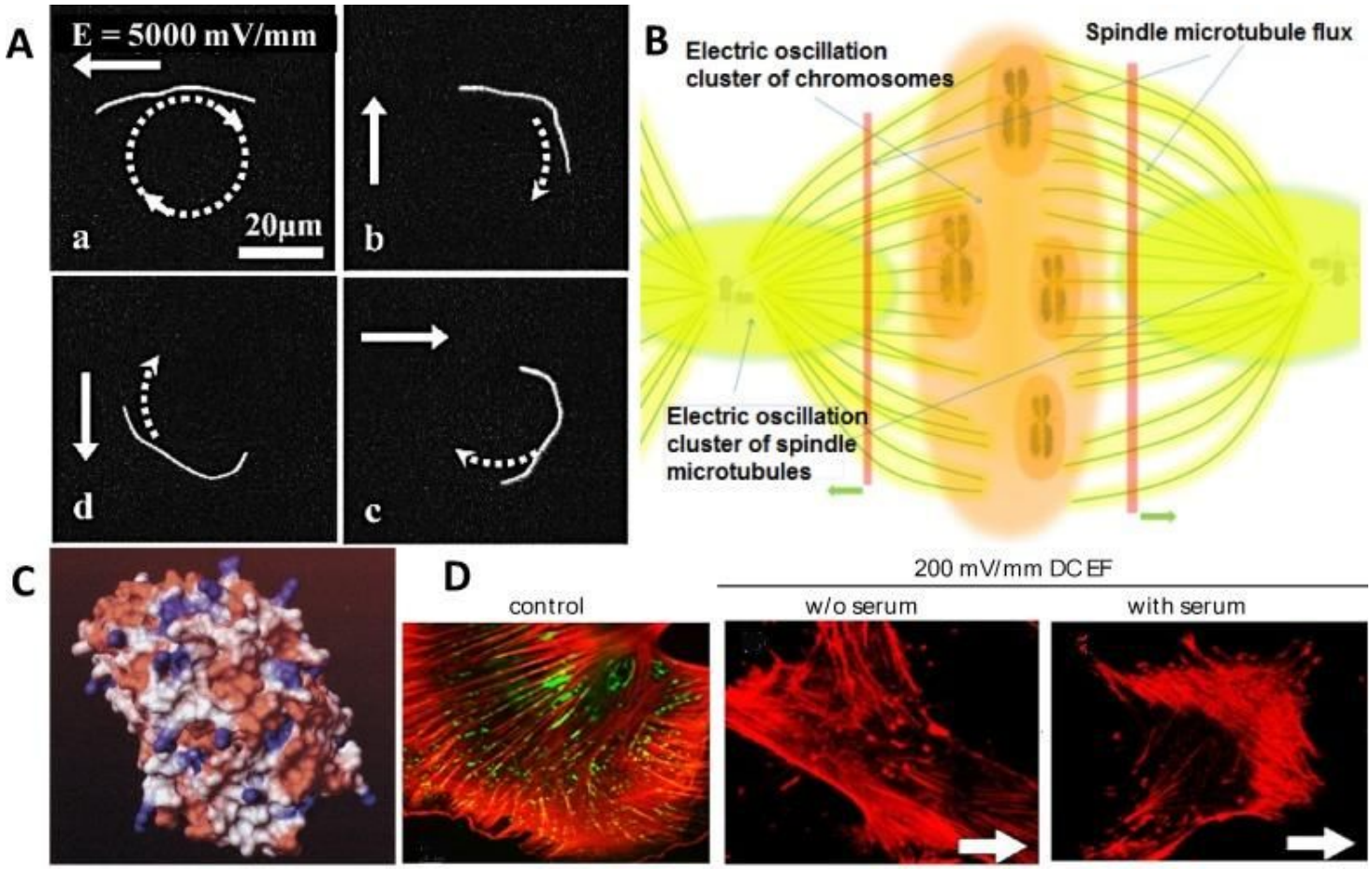

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Fig. 8. (A) Fluorescence image showing circular translocation of kinesin powered microtubule with the application of rotating EF. The continuous application of electrophoretic force rapidly redirects the negatively charged microtubules parallel to the electric field, by deflecting their leading end toward the anode. Reprinted with permission from Ref. [217]. Copyright (2007) American Chemical Society. (B) Schematic showing intracellular EF interactions between chromosome and microtubules, which regulate several nuclear and cellular events during mitosis and meiosis. These EFs are generated under energy excitation through the synchronized electric resonant oscillations of the dipolar structures of super-macromolecular complexes. Reproduced with permission from Ref. [216], ◦ Zhao and Zhan; licensee BioMed Central Ltd. 2012, under Creative Commons Attribution 2.0 International Public License). (C) 3D Protein model shows the electric charge distribution on the surface of the α-tubulin monomer at physiological pH. Red colour indicates positive charge, blue negative, and white neutral areas.

Reprinted from Ref. [225], Copyright ◦ 2002 with permission from Elsevier. (D) Immunofluorescent image showing the disruption of actin stress fibers (labeled in red) in hMSCs after exposure to a 200 mV/mm DC EF for an hour. A noticeable difference in the disassembly of actin filaments can be observed in serum containing media, wherein actin polymerization occurred near the cathodal side and depolymerization happened at the anodal side of the cell. White arrow indicates the direction of the EF applied. Adapted and reproduced from Ref. [37], Copyright ◦ 2009 Biophysical Society, with permission from Elsevier.

Of the cytoskeletal architecture of the cell consisting of microtubules, actin filaments, and intermediate filaments, microtubules are of unique importance since they exhibit several characteristic features that distinguish them from other sub-constituents of the cytoskeleton [230]. Interesting is the fact that microtubules generate a longitudinal electric field around them, under intracellular energy excitation, by the harmonic oscillation of α and β tubulinsubunits that form electric dipoles during microtubule polymerization [231]. In an attempt to manipulate microtubules using externally applied EFs, Kim et al. demonstrated the alignment of kinesin powered microtubules along the field direction by moving their leading end towards the anode [232]. Intriguingly, circular translocation of moving microtubules was achieved by applying rotating EF around the periphery of the device in the same study. Likewise, several published literature have shown that EFs can be successfully employed in prompting the parallel assembly and orientation of microtubules, *in vitro*[233], [234], [235].

Previous investigation even suggests the possibility to selectively steer negatively charged microtubules using perpendicular EF of 0–5000 mV/mm (Fig. 8A) [236]. Contrasting results were also reported previously, wherein suspended microtubules failed to orient in the presence of weak electrophoretic forces [237].

Nevertheless, it is postulated that electric dipoles of actin and tubulin orient along the field direction, thus promoting polymerization parallel to the EF lines. Also, actin polymerization in the direction of EF is the key driving force for electrotaxis or EF guided cell migration. Actin-related proteins 2 and 3 (ARP2/3) complex were shown to promote actin nucleation and polymerization in the direction of applied EF of 50–200 mV/mm, which guided the cathodal migration of neural stem cell derived oligodendrocyte precursor cells (NSCs-OPCs) [131]. Recently, stimulation with highly concerted sub-microsecond EF pulses was shown to elicit coordinated motion of amino acid residues within human PDZ domain of model protein crystals as evidenced by time-resolved X-ray crystallography [238]. These motions are similar to the local and allosteric conformational changes that occur naturally during ligand binding of protein active sites to cell surface receptors.

Such EF stimulated protein mechanics can trigger structural changes within cytoskeletal and intracellular proteins thereby impacting cellular functionality. Altogether, the reliability and agility of feedback to intracellular signaling can be regulated by modifying cytoskeletal pre-stress, which governs tension in cytoskeletal filaments including actin stress fibers and intermediate filaments that extend over long stretches in the cytoplasm. Correspondingly, the electrical forces on the nucleus, can lead to alterations in the structure, folding conformation or kinetics of explicit load-bearing molecules. It can further promote changes in higher-order chromatin assembly, and thereby alter nuclear protein organization, gene transcription, DNA replication or RNAprocessing – all of which dictate cellular functions.

4.4. Surface receptor redistribution

Many cellular processes are triggered and controlled by ligand-receptor binding. The changes in receptor distribution could affect cellular responses by altering the kinetics or affinity of ligand-receptor interactions. These membrane proteins function as signal integrators, which can respond to extracellular ligands, intracellular signal transduction, and changes in transmembrane potential [228]. For instance, integrins and its associated linking proteins, namely vinculin, actinin, and talin, interact with cellular actin filaments to form focal-adhesion complexes (FACs), which are

essential for cell attachment and motility [217], [239]. By theoretical calculations, it was shown that low frequency EFs of physiological magnitude (100 mV/mm) exert mechanical forces of the order of 10^{-15} N on model integrins, which is identical to that produced by sinusoidal fluid shear [240].

This led to the hypothesis that electric fields and fluid shear stresses share a common signal transduction mechanism involving integrin receptors, that can dictate stem cell adhesion and differentiation via mechanical forces. Also, α2β1 integrins have also been implicated in directed cell migration as a result of polarization and clustering by RhoA under the influence of applied EF [241]. Mechanistically, since the plasma membrane has high electrical resistance, both dc and low frequency oscillatory ES are likely to confine at the cell surface rather than penetrating inside the cell. As a result, a majority of ES induced biochemical signal transduction cascades are likely to originate at the cell surface via the redistribution of charged cell surface receptors (CSRs) [227].

The peptide units of protein molecule have several charged groups and structural units possessing electric dipole moments that are susceptible to various types of electrical perturbation. More so, the effect of intense EF experienced by these molecules is greatly enhanced if these molecules are embedded in the lipid bilayer, causing conformational changes leading to alteration in their affinity to ions [242].

The dynamics of CSR redistribution is usually dictated by the two competing phenomena, diffusion and lateral electromigration [243]. It is reported that the exposure to external EF of 100–3000 mV/mm strength induces relative electrophoretic movement of charged membrane proteins and lipids, resulting in the redistribution of these components on the cell exterior [244]. Specifically, application of low EF is widely known to upregulate epidermal growth factor receptor (EGFR) along with cathodal redistribution of EGFRs [245]. In addition to inducing an asymmetric distribution of EGFR, small EF also causes co-localization of membrane lipids and second-messenger signaling molecule ERK ½, leading to the activation of MAPK signaling cascade [110], [246]. However, the redistribution of CSR does not exactly tread the EF lines and depends considerably on the frequency of EF stimulation as well as the surface geometry of the cells [18]. It is also worthwhile to share the prediction of McLaughlin et al. [247] that a mobile, negatively charged CSR will shift towards the positive side of the cell only if its zeta potential is more negative than the zeta potential of the cell surface. If the zeta potential of CSR is less negative than the zeta potential of the cell surface, then the CSR will accumulate towards the negative pole of the cell by electro-osmotic fluid flow [247]. Apart from electrophoretic and electroosmotic driven redistribution, cytoskeletal reorganization is also responsible for EF-induced receptor redistribution [18]. In another example, EF-induced redistribution of the unliganded LDL-R (low density lipoprotein receptor) due to surface electrophoresis is clearly demonstrated by Webb and co-workers [248]. Thus, EF application can induce direct CSR activation and downstream signaling in the absence of extracellular ligands, suggesting a ligand-independent activation phenomenon by EF [244]. The capacity of EF both to cause ligand concentration gradients in the cell surroundings and to promote irregular CSR distribution on the plasma membrane indicate that these two cell signals communicate as a positive-feedback amplification loop. Reiterating, the maximum accumulation of CSRs at the leading edge would be triggered by the highest ligand concentrations, both regulated by the EF gradient [1]. Some possible interpretations for EF induced redistribution of CSRs include the movement or rotation of the cells in the field or by gross membrane

alteration or anisotropic membrane characteristics, and irregular shape of cell surface, relative to the electromigration path or due to the buildup of chemical gradients [142], [243].

4.5. ATP synthesis

The membrane ATPases are understood to absorb defined quanta of electrical energy from oscillating EFs of specific frequency and magnitude. This signal can be used to regulate the activity of membrane proteins, a mechanism named as **'electroconformational coupling'**[249]. When an applied EF reaches the mitochondria membrane, it would augment and accelerate ATP synthesis. Hence, any cellular process limited by energy availability would be enhanced by applied electrical current [250]. The synthesis of ATP in non-respiring sub-mitochondrial particles from rat liver, by exposure to high field strength electric pulses is an apt example to describe how electrical energy is transformed into the chemical bond energy of ATP, by the mitochondrial inner membrane [251]. In fact, ATPases of chloroplasts, thermophilic bacteria and mitochondria have also been activated to biosynthesize ATP from ADP and Pi by exposing to pulsed EF [252]. Both, direct electric currents (ranging from 10 to 1000 μA) as well as alternating EF amplitudes of 250–5000 V/mm (100 Hz) stimulated membrane bound ATP synthesis [253], [254].

It is based on the view that electrostimulation can guide migrating protons to reach the mitochondrial membrane bound H^{1}-ATPases so as to generate ATP. Also, a high level of ATP was released from electro-stimulated cells, possibly through secretory vesicles by exocytosis or particular ATP-transporting systems, such as anion channels, or even through transient electrophoretic membrane damage [37]. The release of intracellular ATP via anion channels to the extracellular compartment was increased 50 fold in electro-stimulated HeLa cells. It is believed that the released ATP may exert mitogenic effects by purinergic receptor stimulation via autocrine and paracrinemechanisms [255], [256].

As it is well-known that the mitochondria, which incorporate a dual membrane structure, are central organelles of energy metabolism, the EF induced transmembrane potential across mitochondrial membranes can affect cellular metabolisms [17]. Presumably, many cell functions such as DNA/RNA/protein biosynthesis and enzymatic activities are stimulated or suppressed by weak oscillating electromagnetic fields [249]. Interestingly, electrostimulation can directly accentuate protein synthesis without effecting DNA metabolism due to the increased availability of free amino acids. This in turn is equally increased due to the stimulated amino acid transport. Similarly, the kinetics of both protein and DNA synthesis was significantly escalated at EF strength of 50 and 75 V, respectively, with a pulse rate of 100 pulses/sec, on cells located at the cathode. Conversely, the exposure of cells to EF intensities greater than 250 V appeared to inhibit both protein and DNA synthesis. Since the protein synthesis intensely depends on adequate ATP levels, increased ATP production due to electrostimulation is yet another significant factor responsible for this phenomenon [257].

Particularly, the relationship between ATP synthesis and actin cytoskeleton is one of the intriguing mechanisms by which the cells sense EF. It has been well reported that intracellular ATP is consumed for the conversion of monomeric G-actin to polymeric F-actin [258]. The EF (200 mV/mm) induced ATP depletion was implicated in the reorganization of actin cytoskeleton and inhibition of linker proteins in electrically stimulated hMSCs [259]. Therefore, from a clinical perspective, regenerative process consumes a lot of ATP as its involvement is critically important for the

functioning of virtually every cell and its synthesis and consumption are proven to be accelerated by means of EF stimulation.

4.6. Heat shock proteins

It has been generally hypothesized that the response of cells to EF mimics a generalized physiological stress response. Hence, it is believed that EFs basically function as stress inducers and induce cellular responses through the activation of stress proteins, such as heat shock proteins (HSPs) [260]. A plausible upregulation of the hsp70 gene is invoked in electrically stimulated cells. This hypothesis can be rationalized in that hsp70 is expressed not only by thermal shock, but also by many other stresses such as pressure, magnetic and shear stresses [261]. Experimental evidences also suggest the participation of heat shockproteins (hsp 27 and hsp 70) in the upregulation of some of the transcription factors that aids in osteogenic differentiation of hMSCs [154]. Even, a relationship was drawn between the electrical conditioning of cells and the extent of HSP70 promoter expression, with the latter varying as a function of the field strength, pulse duration of the stimulation, and exposure time [262].

By its very nature, EF exposures can elicit a heat shock-like response. Unlike heat shock, the effect of ES was not significant at the mRNA transcription level, but more pronounced at the protein level of HSP via the attenuation of proteasomal degradation [263]. In a study by Yanagida et al. EF stimulation caused a 3 to 6-times greater HSP70 expression than thermal heat shock-induced expression [261]. It was quantified that EMF induces the production of hsp70 protein at an energy density of 14 orders of magnitude below that of thermal heat shock [264]. Another important breakthrough is the discovery of an EM field-sensitive nucleotide sequence in the HSP70 promoter. The EF domain is present between 230 and 160 on the HSP70 promoter and consists of three nCTCTn recognition motifs/sequences[265]. The elimination of the 70 bp region of the HSP70 promoter, with its three nCTCTn sequences, ceases the response to EF, but does not affect the response to heat shock [266]. Moreover, EF can influence HSPs not only in terms of gene/protein expression, but also their distribution inside cellular compartments [267]. Considering the effectiveness of ES in activating HSP70 promoter, neural differentiation was achieved by electrically stimulated NeuroD2 expression under the control of HSP70 promoter by Aizawa and team [268]. All these instances collectively suggest that exposure to EF induces the expression of HSPs, which can interact with a variety of transcription factors as well as intrinsic and extrinsic signaling pathways affecting self-renewal, proliferation and differentiation of stem cells.

4.7. Reactive oxygen species

Another important mechanism through which stem cells respond to EF is through reactive oxygen species (ROS) generation and its participation in important signaling pathways [269]. As ROS is predominantly generated by NADPH-oxidase in stem cells, it has been postulated that their activation and expression can in turn be modulated by physical cues [270]. It should be noted that cell response of different cell types is variable to the same biochemical signal, which can be attributed to their particular intracellular redox state [271]. Since every cell type differ in their antioxidative capacity, its sensitivity towards ROS will be distinctly different, which eventually determines the cell fate.

While largely known for its detrimental effects such as DNA damage, protein/lipid oxidation and apoptosis, there are growing literature evidences showing that moderate intracellular ROS in the stem cell population induces them to undergo differentiation [272]. In fact, controlled ROS generation at physiologically moderate levels can facilitate beneficial interaction with other signaling molecules involved in differentiation. The prerequisite of moderate ROS for the activation of MAPK pathways and the consequent signaling cascades of ERK1,2, JNK and p38 has been testified in a number of studies [270]. In fact, a consensus has emerged that hypoxia induced mild rise in ROS mediates proliferation and differentiation of MSCs and PSCs [273], [274]. The stimulation of skeletal muscle cells with physiologically relevant EF led to ROS generation through the discharge of extracellular ATP and incitement of P2Y1 receptors [269]. Furthermore, cardiac differentiation in electrically stimulated hESCs occurs through pathways associated with the transient increase in intracellular ROS level [4].

It is also understood that ROS and other free radical generation, as well as acidification develop at the anode–electrolyte interface, when higher EF intensity is applied in a physiological solution [244]. Altogether, it is assumed that elevated intracellular ROS level in response to EF exposure acts as a signal transducer, which possibly could initiate various differentiation programs [4].

4.8. Lipid rafts

It is widely accepted that biological cells sense external EF through charged molecules, membrane proteins and receptors that polarize under the influence of DCEF [275]. The polarization of membrane proteins and ion channels were hypothesized to activate intracellular signaling pathways, that regulate cell migration. However, recent research has shown that glycolipids on the plasma membrane can redistribute and congregate into nanodomain structures by employing membrane lipids and proteins under an external EF [276]. These nanodomain structures known as lipid rafts are inert to nonionic surfactants and may be the principal sensors of EF within cells. Under an applied EF, the congregation of glycolipids by recruiting membrane lipids and proteins leads to an increase in the raft size, thereby reducing raft motility. The consequent polarization of the raft structures leads to directed cell migration. Recently, Lin et al. demonstrated a frequency dependent raft and cell migration under AC EF [277]. Using experimental and theoretical modeling approaches, their work confirmed that lipid rafts act as mobile nanodomain complexes that polarize and promote directed cell migration in AC EF by sequestering caveolin and integrins on the cell surface. The membrane rafts are also implicated in many biological phenomena such as wound healing, immune response, axonal outgrowths in neural cells and polarization of cell motility during chemotaxis [278].

5. Effects of electrical stimulation *in vivo*

In the light of endogenous electric field mediated wound healing of the epithelial skin tissue, EF triggered cardiac pacing and rhythm, EF modulated bone homeostasis, nerve signal transmission and skeletal muscle contractility, exogenous EF has been applied as a biomimicry tool for regulating tissue behavior and regeneration [199]. In this concluding section, a brief summary of the effects of exogenous or external electrical stimulation in various tissues is presented. The piezoelectricity of bone and its role in bone remodeling was first established by Fukada and Yasuda [151]. The presence of collagen fibrils in the framework of the bone tissue confers piezoelectricity, which was exploited in their pioneering work on the accelerated fracture reunion of rabbit femur by constant current stimulation for 3 weeks [279].

The results were rationalized on the basis of the reverse piezoelectric effect wherein an applied voltage causes compression of the bone tissue. Concerning electrical stimulation for bone healing, it has been reported that the application of electronegative potentials cause bone compression leading to bone formation, while electropositive potentials induce tension in the bone leading to bone resorption [280]. Subsequently, direct current electric stimulation has been tested in clinical trials for spinal fusion surgeries. Commercial implantable spinal fusion stimulators (SpF, Biomet Spine) with 40–60 μA DC current output are available to aid in union of injured spines. In a randomized controlled clinical trial, 81% success rate of posterior spine fusion was recorded using ES in comparison to 54% in conventionally treated spine surgeries [281]. Recently, an electronic device was coupled to dental implants for providing biphasic electrical stimulation with a current density of 20 μA/cm^2, which promoted osseointegration of dental implants with the mandibular bone in Canine models [282]. Likewise, the electrical stimulation of neural tissue is performed at specific sites in the brain in order to treat neurological and neuromuscular disorders. Particularly, deep brain stimulation (DBS) is employed in cases of extreme neurological conditions like Parkinson's disease, tremors and seizures. The stimulation parameters vary with the anatomical region of the brain being stimulated and the treatment requirements. Generally, in DBS, the subthalamic nucleus (STN) and globus pallidus interna (GPi) parts of the brain are treated with typical parameters of 1–3.5 V, pulse duration of 60–210 μs and frequency range of 130–185 Hz [283].

On similar lines, transcranial direct current stimulation (tDCS) is a non-invasive technique, which utilizes a constant current source of 0–4 mA to trigger the brain cortex to revive neuronal cell activity, following brain stroke [284]. As necessary for treatment of brain stroke, directed migration of transplanted human neural stems cells (hNSCs) in rat brain was achieved by a programmed intermittent EF of 0–300 mV/mm [285]. Recently, a new method for stimulating selective regions of the brain without affecting neuronal activityin the vicinity was developed by applying temporally interfering EFs [286]. Further, electroacupuncture was used to stimulate rat and human brains at specific points, which led to the release of mesenchymal stem cells into the peripheral blood predominantly from adipose tissue [287]. It is currently being tested pre-clinically and clinically for stroke rehabilitation and seizures by functional electrical stimulation exposure [107]. Another potential application of neural electrical stimulation is the function of a pain-killer or analgesic during specific surgical procedures. Transcutaneous electrical nerve stimulation (TENS) is the transmission of electric current to superficial nerves via the skin to function as an anesthetic during dental procedures and analgesic against acute or chronic maxillofacial pain after the procedure [288].

In similar way, the application of electrical stimulation for cardiovascular applications is also discussed. Cardiac pacing and defibrillation are among the most important heart disease treatment functions of electrical stimulation. Cardiac arrhythmias or irregular heartbeat, leading to speeding up or slowing down of the heart rate is treated by surgical implantation of a cardiac pacemaker in the chest close to the heart. Typical operating parameters of a cardiac pacemaker are electrical pulses of 5 mA/cm^2 current density, 2 ms duration and a frequency identical to the normal heart rate of 72 beats per minute (bpm) from a pulse generator of 5–8 V [289]. The electrodes are placed on the myocardium of atria or ventricles or both for dual pacing. In the case of cardiac pacing, an electric field of 100 mV/mm is sufficient for stimulating the heart muscle, while defibrillation uses a much higher field of 800 mV/mm for reviving the heart, following cardiac arrest due to ventricular fibrillation.

By functional electrical stimulation similar to cardiac pacing, rhythmic aortal contractions were recorded in mice with intensities proportional to the applied voltage and at native (1.7 Hz) as well as accelerated heart rates (5 Hz).

These effects of EF were nullified in the presence of α-adrenergic antagonist drug, phentolamine [290]. Penultimately, chronic square wave pulse electrical stimulation of the lower leg skeletal muscles in Sprague-Dawley rats enhanced the density of blood capillaries by ~30% along with a concomitant increase in the expression of VEGF and Angiotensin II, testing the positive effect of controlled EF mediated angiogenesis [291]. Lastly, a clinical trial recorded an enhancement in angiogenesis as well as marked reduction in wound size, following degenerate wave pulse stimulation in a skin wound healing model in humans [292]. All the pre-clinical and clinical studies presented in this section and Table 4 are instances that exemplify the critical role of electrical stimulation in the tissue regeneration of bone, dental, neural, cardiac, blood vasculature, skin and wound healing. Also, a parallel may be drawn between the EF induced stem cell proliferation, migration and differentiation (section 4), *in vitro* and the electrical stimulation effects, *in vivo*(section 6).

With these cues, it seems a certain possibility to apply electrically manipulated stem cells for tissue engineering and regenerative medicine with considerable success. A detailed investigation of the specific *in vitro* and *in vivo* mechanisms that operate under particular EF exposure conditions would help design stimulation protocols and standardize them for therapeutic applications.

Table 4. Summary of electrical stimulation effects, *in vivo*.

Electrical stimulation	Stimulation parameters	Animal mode and tissue type	Outcome of the study	Reference
Direct current (DC) EF	DC current for 3 weeks	Rabbit femur	Accelerate reunion of fractured bones	Yasuda et al., 1953 [279]
Biphasic electrical current (BEC) stimulation	20 μA/cm^2, 125 μs and100 Hz for 3–5 weeks	Canine mandible	Enhanced osseointegration of dental implant with the mandibular bone upon electrical stimulation of the tissue surrounding the implant	Song et al., 2009 [282]
Direct current implantable spinal fusion stimulator (SpF by Biomet Spine)	40–60 μA	Lumbar spinal fusion in humans	Clinical study revealed 81% fusion success rate with DC stimulation of injured posterior spines compared to 54% in conventionally treated subjects	Kane WJ. 1988 [281]
Deep brain stimulation (DBS)	1–3.5 V, 60–210 μs, 130–185 Hz	Subthalamic nucleus (STN) and globus pallidus interna (GPi) parts of the brain	DBS is employed for treating movement disorders including Parkinson's disease, tremor and other neurological disorders. Stimulation parameters vary with anatomical region and requirement.	Kuncel et al., 2004 [283]
Functional EF stimulation (similar to seizures)	30 Hz, 250 μs,, 3 mA at 10 min/day for 14 days	Cerebral artery occlusion models of Sprague-Dawley rats	Neural cell precursor proliferation and expression of fibroblast growth factor (FGF) and epidermal growth factor (EGF) in the brains of rats	Xiang et al., 2014 [107]

Transcutaneous electric nerve stimulation (TENS)	Pulsed AC or DC current from a 9 V source	Electric signals are transferred to superficial nerves via the skin surface	Electrical stimulation acts as analgesic for treatment of acute and chronic pain during dental procedures and pain in the maxillofacial region	Kasat et al., 2014 [288]
Cardiac pacing and defibrillation	5-8 V, 2 ms, 5 mA/cm^2, ~72 beats/min	Atrial/ventricular or dual pacing (100–200 mV/mm) and sensing; 800 mV/mm for defibrillation	Treatment of cardiac arrhythmia or irregular heart beat by stimulating atrial or ventricular myocardium; Defibrillation is the delivery of electric current following cardiac arrest due to ventricular fibrillation	Malmivuo J et al., 1995 [289]
Pulsed electrical stimulation	0.25–10 V, 2 ms, 1.7 (normal heart rate) and 5 Hz	Aorta of Sprague-Dawley rats	Rhythmic aortal contractions were recorded in intensities proportional to applied voltage and at a native heart rate. These effects were suppressed by α-adrenergic antagonist drug, phentolamine.	Sahibzada et al., 2015 [290]
Square wave pulse stimulation	3 V, 0.3 ms, 10 Hz at 8 h/day for 1 week	Lower leg muscle tissues of Sprague-Dawley rats	Chronic stimulation for 1 week led to 15–30% increase in the density of blood capillaries coupled with high VEGF and Angiotensin II in the stimulated muscle tissues.	Amaral et al., 2001 [291]
Degenerate wave electrical stimulation	4 μA, 60 ms, 20–40 V, 60 Hz at time points over 3 weeks	5 mm punch biopsies in the inner upper arm of humans	Electrical stimulation induced angiogenesis (VEGF) and decreased wound size in acute wound healing model of human skin	Ud-Din et al., 2015 [292]
Intermittent EF	0-300 mV/mm for 10 h per day	Rat brain post transplantation of hNSCs	EF directed migration of transplanted hNSCs in the rat brain	Feng et al., 2017 [285]
Temporally interfering EF	10 Hz from 2.01 to 2 kHz interfering frequencies	Neurons in the hippocampus regions of mice brain	Selective firing of hippocampal neurons without affecting cortical neurons in the vicinity	Grossman et al., 2017 [286]

6. Conclusions

In summary, the present review uncovers a valuable glimpse into an unexplored domain of stem cell manipulation via electrical cues. While discussing the EF stimulation on stem cell response, the influence on other cell types are also mentioned. As far as the biophysical mechanisms are concerned, it has been largely emphasized that a combination of multiple signal transduction pathways, cytoskeletal reorganization and actin distribution and surface receptor redistribution operate under exogenous EF. Also, the up/downregulation of Ca^{+2}transients, ATP synthesis, heat shock proteins and reactive oxygen species can mediate EF modulated stem cell response. An integrated approach to develop a qualitative and quantitative understanding of the stem cell differentiation through different lineages under the influence of electrical stimulation, remains to be explored in future.

Both endogenously generated and exogenously applied EF have pivotal role in evoking stem cell response and it represents a remarkable tool for translational applications in bone/cardiac/neural tissue engineering and regeneration. However, proper identification and understanding of the critical field parameters that produce desired biological effects in stem cells is of prime importance in establishing safe and therapeutically relevant levels of EF exposure. The existing literature suggest

that the modulation of stem cell proliferation, migration and differentiation can be achieved by mimicking endogenous bioelectric fields in terms of the mode of stimulation (direct, pulsed, combined electromagnetic field etc.), the EF pattern, intensity and/or duration of a specific signal. However, several key questions related to defining the specific mechanisms of stem cell activation by external electrical stimuli, remain to be answered in order to facilitate the translation of these findings in basic research to clinical applications. More importantly, significant lessons are to be learnt by correlating the EF parameters and level of tissue/substrate conductivity required to trigger differentiation of choice, if these variants function in harmony to govern stem cell behavior. Here, the synergistic interaction among all the parameters/factors leading to cell functionality modulation is to be firmly established in future. Finally, this review emphasizes the clinical therapeutic potential of electromagnetic stimulation for bone healing, deep brain stimulation, cardiac pacing and defibrillation. Taken together, the bench to bed translation of the concept of 'EF stimulation of stem cell niche' in the context of tissue engineering and regenerative medicine is another aspect that needs further investigation.

Although a multitude of *in vitro*studies together with considerable preclinical studies are being reported in literature and discussed in this review, translational research would require more pre-clinical studies followed by thoughtful clinical trials [293], [294].

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Appendix. List of abbreviations

EF
electric field

DCEF
direct current electric field

PEF
pulsed electric field

AC EF
alternating electric field

CC
capacitive coupling

IC
inductive coupling

PEMF

pulsed electromagnetic field

ES
electrical stimulation

t-DCS
transcranial direct current stimulation

TENS
transcutaneous electrical nerve stimulation

PPy
polypyrrole

PANI
polyaniline

PT
polythiophene

SS
stainless steel

ITO
Indium tin oxide

CNTs
carbon nanotubes

hMSCs
human mesenchymal stem cells
NSCs
neural stem cells

hESCs
human embryonic stem cells

hiPSCs
human induced pluripotent stem cells

hASCs
human adipose tissue-derived stem cells

TMP
transmembrane potential

FGF
fibroblast growth factor

EGF
epidermal growth factor

EGFR
epidermal growth factor receptor

IGF
insulin-like growth factor

VEGF
vascular endothelial growth factor

CaM
calcium calmodulin

NOS
nitric oxide synthase

cGMP
cyclic guanosine monophosphate

Erk
extracellular signal regulated kinase

CREB
cAMP response element binding protein

MAPK
mitogen activated protein kinase

FAK
focal adhesion kinase

JNK
c-jun N-terminal kinase

PTEN
phosphate and tensin homolog

EBs
embryoid bodies

GNPs
gold nanoparticles

ROS
reactive oxygen species

CSRs

cell surface receptors

ER
endoplasmic reticulum

VGCCs
voltage gated calcium channels

SACCs
stretch activated calcium channels

NCX
Na + -Ca2+ exchanger

GPCR
G-protein coupled receptor

IP3
inositol 1,4,3-triphosphate

[Ca2+]i
intracellular calcium

ATP
adenosine triphosphate

HSPs
heat shock protein

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